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(71) Applicant *(for all designated States except US)*: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : BASS, Steven, H. [US/US]; 1355 Sierra Street, Redwood City, CA 94061 (US). CUNNINGHAM, Brian, C. [US/US]; 24 Olive Avenue, Piedmont, CA 94611 (US). FUH, Germaine [CN/US]; 2610 - 39th Avenue, San Francisco, CA 94116 (US). LOWMAN, Henry, B. [US/US]; 205 Falcon Way, Hercules, CA 94547 (US). MATTHEWS, David, J. [GB/US]; 1527 Noriega Street, San Francisco, CA 94122 (US). WELLS, James, A. [US/US]; 1341 Columbus Avenue, Burlingame, CA 94010 (US)

(74) Agents: BENSON, Robert, H. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

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(54) Title: METAL ION MEDIATED RECEPTOR BINDING OF POLYPEPTIDE HORMONES

(57) Abstract

Novel methods are disclosed for modulating the action of polypeptide hormones on mammalian cells, organs or whole mammals. Polypeptide hormone action is controlled by effecting the binding specificity of the polypeptide hormone for distinct receptors. The specificity for the receptor is mediated by the ability of a metal ion to complex between the polypeptide hormone and its receptor. Soluble variants of the hormone receptor may be used to modulate the action or serum half-life of the complexed polypeptide hormone. An example of such a polypeptide hormone system is human growth hormone (hGH) wherein receptor specificity is modulated by the metal cofactor zinc. Under low zinc conditions, hGH preferentially binds to human growth hormone receptor or binding protein, under high zinc conditions, hGH preferentially binds to human prolactin receptor or soluble prolactin receptor variants. This is the first indication that a metal ion can mediate a direct interaction between a polypeptide hormone and an extracellular receptor or binding protein.

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METAL ION MEDIATED RECEPTOR BINDING OF POLYPEPTIDE HORMONES

BACKGROUND OF THE INVENTION

Field of the Invention

Described are novel methods for controlling the response of a cell, organ, or an animal, to polypeptide hormones by inserting or deleting a metal ion cofactor binding site in the polypeptide hormone-receptor complex; either by creating polypeptide hormone variants, or in specific cases variant receptor-like binding proteins.

Description of the Background Art

Metal ions such as zinc have been shown to be useful in the prolonged parenteral release of somatotropins in an oil formulation (EP 177,478, published 04.10.84; EP 343,696, published 29.11.89). Similiar slow release formulations of bovine growth hormone complexed with metal ion in an oil vehicle have been shown (EP 216,485, published 01.04.87). Metal ions have been used to recover somatotropin from dilute aqueous solutions by forming a precipitate (EP 277,043, published 03.08.88). Prolactin has been examined as a regulatory hormone for zinc uptake by the prostate gland (Leake et al., J. of Endocrinology 102(1), p73-76, 1984). Zinc deficiency has been associated with a tendency to hyperprolactinemia (Koppelman, Medical Hypotheses, 25(2), p65-68, 1988). A review of the zinc requirement in humans can be found in Prasad (Special Topics in Endocrinology and Metabolism, vol 7, p45-76, 1985).

Human growth hormone (hGH) participates in much of the regulation of normal human growth and development. This 22,000 dalton pituitary hormone exhibits a multitude of biological effects including linear growth (somatogenesis), lactation, activation of macrophages, insulin-like and diabetogenic effects among others (Chawla, R, K. (1983) Ann. Rev. Med. 34, 519; Edwards, C. K. et al. (1988) Science 239, 769; Thorner, M. O., et al. (1988) J. Chin. Invest. 81, 745). Growth hormone deficiency in children leads to dwarfism which has been successfully treated for more than a decade by exogenous administration of hGH. There is also interest in the antigenicity of hGH in order to distinguish among genetic and post-translationally modified forms of hGH (Lewis, U. J. [1984] Ann. Rev. Pyhsiol. 46 , 33) to characterize any immunological response to hGH when it is administered clinically, and to quantify circulating levels of the hormone.

hGH is a member of a family of homologous hormones that include placental lactogens, prolactins, and other genetic and species variants or growth hormone (Nichol, C. S., et al. (1986) Endocrine Reviews 7, 169). hGH is unusual among these in that it exhibits broad species specificity and binds monomerically to either the cloned somatogenic (Leung, D. W., et al. [1987] Nature 330, 537) or prolactin receptor (Boutin,

J. M., et al. [1988] Ce; 53, 69). The cloned gene for hGH has been expressed in a secreted form in Eschericha coli (Chang, C. N., et al. [1987] Gene 55, 189) and its DNA and amino acid sequence has been reported (Goeddel, et al. [1979] Nature 281, 544; Gray, et al. [1985] Gene 39, 247). The three-dimensional structure of hGH is not available. However, the three-dimensional folding pattern for porcine growth hormone (pGH) has been reported at moderate resolution and refinement (Abdel-Meguid, S. S., et al. [1987] Proc. Natl. Acad. Sci. USA 84, 6434). Human growth hormone's receptor and antibody epitopes have been identified by homolog scanning mutagenesis (Cunningham et al, Science 243: 1330, 10 March 1989). The structure of novel amino terminal methionyl bovine growth hormone containing a spliced-in sequence of human growth hormone including histidine18 and histidine 21 has been shown (U.S.Patent 4,880,910).

Human growth hormone (hGH) causes a variety of physiological and metabolic effects in various animal models including linear bone growth, lactation, activation of macrophages, insulin-like and diabetogenic effects and others (R. K. Chawla *et al., Annu. Rev. Med.* **34**, 519 (1983); O. G. P. Isaksson *et al., Annu. Rev. Physiol.* **47**, 483 (1985); C. K. Edwards *et al., Science* **239**, 769 (1988); M. O. Thorner and M. L. Vance, *J. Clin. Invest.* **82**, 745 (1988); J. P. Hughes and H. G. Friesen, *Ann. Rev. Physiol.* **47**, 469 (1985)). These biological effects derive from the interaction between hGH and specific cellular receptors. Only two different human receptors have been cloned, the hGH liver receptor (D. W. Leung *et al., Nature* **330**, 537 (1987)) and the human prolactin receptor (J. M. Boutin *et al., Mol. Endocrinol.* **3**, 1455 (1989)). However, there are likely to be others including the human placental lactogen receptor (M. Freemark, M. Comer, G. Korner, and S. Handwerger, *Endocrinol.* **120**, 1865 (1987)). These homologous receptors contain a glycosylated extracellular hormone binding domain, a single transmembrane domain and a cytoplasmic domain which differs considerably in sequence and size. One or more receptors are assumed to play a determining role in the physiological response to polypeptide hormones.

One of the best characterized hormone binding proteins is the growth hormone binding protein (GHBP). This GHBP is the extracellular domain of the GH receptor which circulates in blood and functions as a GHBP in several in several species (Ymer SI, Herington AC, Mol Cell Endocrinol (1985) 41:153; Smith WC, Talamantes F, Endocrinology (1988) 123:1489-94; Emtner M, Roos P, Acta Endocrinologica (Copenh) (1990) 122,3:296-302). GHBP in humans has also been described (Baumann G, Stolar MW, Amburn K, Barsano CP, DeVries BC, J Clin Endocrinol Metab (1986) 62:134-141.; Herington AC, Ymer S, Stevenson J, J Clin Invest (1986) 77:1817-1823). DNA encoding human GHBP is described in PCT publication number WO 88/09818, published 15 December 1988.

SUMMARY OF THE INVENTION

Novel methods are disclosed for modulating the action of polypeptide hormones on mammalian cells, organs or whole mammals. Polypeptide hormone action is controlled by effecting the binding specificity of the polypeptide hormone for distinct receptors. The specificity for the receptor is mediated by the ability of a metal ion to bind as part of the hormone-receptor complex and thus to further determine receptor binding specificity. Soluble variants of the hormone receptor may be used to modulate the action or serum half-life of the polypeptide hormone. An example of such a polypeptide hormone system is human growth hormone (hGH) wherein receptor specificity is modulated by the metal cofactor zinc. Under low zinc conditions, hGH preferentially binds to human growth hormone receptor or growth hormone binding protein; under high zinc conditions, hGH preferentially binds to human prolactin receptor or soluble prolactin receptor variants. This is the first indication that a metal ion can mediate a direct interaction between a polypeptide hormone and an extracellular receptor or binding protein.

Novel human polypeptide hormone variants and hormone binding protein variants having therapeutic utility are disclosed: the variants may have a metal ion binding site deleted or inserted. Among the variants having a metal ion binding site deleted are those hormone variants having the ability to preferentially bind to specific receptors as a function of the absence of a zinc binding site. Specifically, human growth hormone variants have histidine$_{21}$ of native human growth hormone replaced by an amino acid other than histidine glutamate, aspartate or cysteine, more specifically, the human growth hormone variant wherein histidine$_{21}$ is replaced by alanine. In addition, the human growth hormone variants may replace histidine$_{18}$ and glutamate$_{174}$ of native human growth hormone with an amino acid other than histidine, glutamate, aspartate or cysteine, more specifically it is replaced by alanine. Another human polypeptide hormone variant is human placental lactogen variant wherein histidine$_{18}$, histidine$_{21}$ or glutamate$_{174}$ of native human placental lactogen is replaced by an amino acid other than histidine, glutamate, aspartate or cysteine, more specifically with alanine.

Disclosed are a mammalian growth hormone variant, excluding human growth hormone, wherein the amino acid corresponding to human growth hormone amino acid histidine$_{18}$, histidine$_{21}$ or glutamate$_{174}$ is replaced by an amino acid other than histidine, glutamate, aspartate or cysteine, more specifically with alanine. Other hormone variants are human growth hormone variants comprising arginine$_{64}$ and aspartate$_{171}$ substituted by alanine; and human growth hormone variants comprising lysine$_{168}$ and glutmate$_{174}$ substituted by alanine; human growth hormone variants comprising lysine$_{172}$ and glutmate$_{174}$ substituted by alanine. Described are DNA sequences encoding the human growth hormone variants, specifically those DNA sequences wherein said variant contains alanine in place of histidine$_{18}$, histidine$_{21}$ and glutamate$_{174}$. Further described is an

expression host transformed with a DNA sequence selected from the group consisting of a DNA sequence encoding a growth hormone variant wherein $histidine_{21}$ of human growth hormone is replaced by an amino acid other than glutamate, aspartate or cysteine and a DNA sequence encoding a growth hormone variant wherein said variant contains alanine in place of $histidine_{18}$, $histidine_{21}$ and $glutamate_{174}$.

Additionally disclosed is a method of modifying a mammalian polypeptide hormone-receptor complex containing a metal ion binding site wherein the presence of a metal ion in the metal ion binding site determines the hormone's affinity for the mammalian hormone receptor comprising replacing a histidine, glutamate, aspartate or cysteine amino acid in a mammalian polypeptide hormone or receptor that chelates the metal ion to the mammalian polypeptide hormone-receptor complex, with another amino acid to prepare a variant hormone or receptor that is reduced in its ability to chelate the metal ion. The metal ion may be zinc, iron, nickel, copper, magnesium, manganese, cobalt, calcium or selenium, most preferably zinc. Preferably, the mammalian polypeptide hormone may be growth hormone or placental lactogen. The hormone receptor may be growth hormone receptor, prolactin receptor, placental lactogen receptor or a serum binding protein with similar receptor properties, for example, growth hormone binding protein.

Further described is a method of stimulating a lactogenic response in a non-human mammal comprising administering to the mammal a therapeutically effective amount of a mammalian growth hormone wherein said mammalian hormone amino acid sequence contains amino acids corresponding to human growth hormone amino acids $histidine_{18}$, $histidine_{21}$ and $glutamate_{174}$, and maintaining a physiological zinc ion concentration required for said mammalian growth hormone to bind to prolactin receptor wherein a lactogenic response is elicited, preferably the total physiological zinc ion concentration is maintained between about 0.5 and 100.0 μmolar. Also described is a method of stimulating a lactogenic response in a human comprising administering to the human a therapeutically effective amount of human growth hormone while maintaining a physiological zinc ion concentration required for said human growth hormone to bind to prolactin receptor wherein a lactogenic response is elicited, preferably the total physiological zinc ion concentration is maintained between about 0.50 and 100.0 μmolar. Additionally described is a method of stimulating a somatogenic response in a human comprising administering to the human a therapeutically effective amount of a human growth hormone variant in which the zinc binding site required for human growth hormone binding to prolactin receptor has been deleted.

Described is a method of screening for variants of a mammalian polypeptide hormone thought to contain a metal ion binding site wherein the presence of a metal ion in the metal ion binding site determines said hormone's affinity for a hormone receptor in a mammal comprising incubating a solution containing a chelating agent and a

mammalian polypeptide hormone variant suspected of containing a metal ion binding site; then contacting the incubated mammalian polypeptide hormone with a hormone receptor; and finally, detecting the formation of a polypeptide hormone-receptor complex. This method may use a metal ion selected from the group consisting of zinc, iron, nickel, copper, magnesium, manganese, cobalt, calcium or selenium. Preferably, the variant mammalian polypeptide hormone may be a variant of growth hormone or placental lactogen. The mammal may be any mammal, preferably selected from human, bovine, porcine, ovine, equine, feline, canine and rodentia.

Also described is a mammalian prolactin binding protein variant comprising soluble prolactin binding protein. Preferably, the soluble prolactin binding protein is human prolactin binding protein. One variant of the human prolactin binding protein has $histidine_{188}$ replaced by an amino acid other than histidine, glutamate, aspartate or cysteine, preferably by alanine. Also described is a mammalian growth hormone binding protein wherein another amino acid is inserted in place of an amino acid corresponding to $asparagine_{218}$ in human growth hormone binding protein that results in the ability to bind a metal ion, preferably zinc. The inserted amino acid is preferably histidine, glutamate, asparate and cysteine. The most prefered form is human growth hormone binding protein. These GHbP agents may be incorporated into a pharmaceutical formulation comprising mammalian growth hormone, mammalian growth hormone binding protein and zinc, wherein the mammalian growth hormone binding protein contains an amino acid substitution with histidine, glutamate, aspartate or cysteine creating a zinc binding site. A preferred formulation contains human growth hormone binding protein wherein $asparagine_{218}$ has been replaced by histidine.

Described is a DNA sequence encoding the human growth hormone variant of human growth hormone wherein the variant contains alanine in place of $histidine_{18}$, $histidine_{21}$ and $glutamate_{174}$. Also described is a DNA sequence encoding soluble human prolactin receptor wherein the human prolactin receptor encoded contains an amino acid substitution at $histidine_{188}$, preferably alanine. Further described is a DNA sequence encoding human growth hormone binding protein wherein $asparagine_{218}$ is replaced by an amino acid selected from histidine, glutamic acid, asparatic acid and cysteine. These DNA sequences may be incorporated into an expression system. The expression host may be transformed with a DNA sequence selected from the group consisting of: 1) a DNA sequence encoding a growth hormone variant wherein $histidine_{21}$ of human growth hormone is replaced by an amino acid other than glutamate, aspartate or cysteine; 2) a DNA sequence encoding a soluble human prolactin receptor; 3) a DNA sequence encoding a soluble human prolactin receptor which contains an amino acid substitution at $histidine_{188}$ other than glutamate, aspartate or cysteine; 4) a DNA sequence encoding a soluble human prolactin receptor wherein $histidine_{188}$ is replaced by

alanine; and, 5) a DNA sequence encoding a human growth hormone binding protein wherein asparagine$_{218}$ is replaced by histidine.

Brief Description of the Drawings

Figure 1A. Diagram of plasmid phPRLbp(1-211) which directs secretion of the hPRLbp into the periplasm of *E. coli*. Genes are indicated by arrows, replication origins by circles, and restriction sites used in the construction are indicated.

Figure 1B. Coomassie blue stained SDS-PAGE (12.5 percent; ref. 27) of purified hPRLbp. Lanes 1-5 are: 1) an *E. coli* periplasmic fraction, 2) the $(NH_4)_2SO_4$ precipitate, 3) the protein after hGH affinity chromatography, 4) the wash just before elution of hPRLbp, and 5) molecular weight standards (ranging from 14 to 97 kD), respectively.

Figure 2. Binding of [^{125}I]hGH to the hPRLbp in the presence of 0.1 percent BSA, 140 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris (pH 7.5) and variable concentrations of total $ZnCl_2$. A fixed 1:1 ratio of hGH and hPRLbp (0.01 nM final) was incubated 16 h in the presence of the indicated concentration of $ZnCl_2$ and the bound [^{125}I]hGH was immunoprecipitated using affinity purified rabbit polyclonal antibodies directed against the hPRLbp.

Figure 3. Equilibrium dialysis for binding of $^{65}Zn^{2+}$ to the hGH•hPRLbp complex.

Figure 4. Proposed Zn^{2+} binding site on hGH that mediates binding to the hPRLbp. Helical wheel projections show the amphipathic character of helix 1 and 4 with polar (shaded) and charged residues (blackened) on one face of the helix and non-polar (open) on the other. The positions of the putative zinc binding ligands, His18, His21, and Glu174, which are involved in binding hGH to the hPRLbp are shown (★). The region where hGH binds to the hGHbp is defined roughly by the shaded circle. Residues marked by the symbols •, ●, ⬤ and ○ represent sites where alanine mutations in hGH cause reductions of 2- to 4-fold, 4- to 10-fold, greater than 10-fold, or 4-fold increase in binding affinity for the hGHbp, respectively.

Figure 5. The amino acid sequence of the extracellular domain of the human prolactin and human growth horman receptor as they are purified after expression in E. coli. Human growth hormone binding protein (shghr) (Seq. ID 1) and human prolactin binding protein (shprlr) (Seq. ID 2) are illustrated. Each binding protein has had the transmembrane region removed. "*" indicates identical amino acids in both sequences.

Figure 6. Competition between hGH and hPRL binding proteins for binding to [^{125}I]hGH. The inset plot shows the data reformulated in a Scatchard plot to calculate of the K_D (68 pM) between hGH and the hPRL bp.

Figure 7. Structural model of hGH based on a folding diagram for pGH determined from a 2.8 Å resolution X-ray structure. Panel A shows a functional map of the hPRLbp epitope and Panel B shows that determined previously for the hGH bp. The symbols (•, ●, ⬤ and ⬤) represent sites where alanine substitutions cause a 2- to 4-fold, 4- to 10-fold, 10-fold to 80-fold, or >80-fold reductions in binding affinity, respectively, for

each receptor binding domain. The ○ in the hGHbp epitope (Panel B) represents the position of E174A that causes greater than a 4-fold increase in binding affinity. Panel C shows sites where alanine mutants reduce binding affinity by ≥10-fold for hPRLbp (□) or ≥5-fold for the hGHbp (■) without affecting substantially the binding to the hGHbp or hPRLbp, respectively. The (▲) symbols show sites where alanine mutants disrupt binding to both receptors by ≥10-fold.

Figure 8. (Seq ID 3) The entire DNA sequence of pB0475 together with the hGH amino acid sequence.

Description of the Preferred Embodiments

There was a need to understand the molecular basis for the pleiotropic receptor binding properties of hGH, therefore, we undertook a systematic analysis of the binding determinants between hGH and its cloned liver receptor (B. C. Cunningham, P. Jhurani, P. Ng, and J. A. Wells, *Science* **243**, 1330 (1989); B. C. Cunningham and J. A. Wells, *Science* **244**, 1081 (1989)). In the present invention, we extended this approach to the hPRL receptor, and discovered that Zn^{2+} is required for tight binding of hGH to the hPRL receptor, but not for binding to the hGH receptor. Moreover, the ability of a metal ion to determine receptor binding specificity indicates that a new mechanism of polypeptide hormone regulation has been discovered.

The molecular details of the hGH-receptor-zinc interaction was experimentally determined using large amounts of the extracellular binding domain of the human prolactin receptor (hPRLbp) as a secreted protein from Escherichia coli. Upon optimizing the binding reaction between hGH and the purified hPRLbp, we discovered that the binding affinity of hGH for the hPRLbp is increased about 8,000-fold (K_D of 270 nM to 0.033 nM) by addition of 50 μM $ZnCl_2$.

Prior to the present invention, there were no known examples of a polypeptide hormone's receptor binding specificity being determined by the presence of a metal ion complexed with the polypeptide hormone and receptor. The ability to change the binding specificity of polypeptide hormones, and therefore their physiological effects, permits the therapeutic control of hormone responses previously not possible. An example of this specificity is the complexing of zinc with human growth hormone and its receptors resulting in a change in relative receptor binding specificity from the human growth hormone receptor (somatogenic response in lower zinc) to the human prolactin receptor (lactogenic response in higher zinc).

A. Definitions

1. <u>Polypeptide hormone</u> may be any amino acid sequence produced in a first cell which binds specifically to a receptor on the same cell for autocrine hormones, or on a second cell type for non-autocrine hormones, and causes a physiological response characteristic of the receptor-bearing cell. Among such polypeptide hormones are cytokines, lymphokines, neurotrophic hormones and adenohypophyseal polypeptide hormones such

as growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, thyrotropin, chorionic gonadotropin, corticotropin, α or β - melanocyte-stimulating hormone, β–lipotropin, γ-lipotropin and the endorphins; hypothalmic releasing hormones such as corticotropin-releasing factor, growth hormone release-inhibiting hormone, growth hormone-releasing factor; and other polypeptides hormones such as insulin, insulin-like-growth factors I and II, and atrial natriuretic peptides A, B or C.

2. Metal Ion Cofactors may be any divalent metal ion which will complex with a polypeptide hormone and/or receptor and increase or decrease affinity between hormone and receptor. Among the preferred metal ions are zinc, iron, nickel, copper, magnesium, manganese, cobalt, calcium or selenium. The metal ions may be any physiological acceptable salt, such as chloride, phosphate, acetate, nitrate and sulfate.

3. Variant Polypeptide Sequence Notation defines the actual amino substitutions in the mutant polypeptides of the present invention, as illustrated in Table 2. For a variant, substitutions are indicated by a letter representing the original amino acid, a number indicating the amino acid position in the polypeptide, and second letter indicating the substituted amino acid.. Therefore, each substitution is represented by a letter followed by a number which is followed by a letter. For example, H18A in Table 2, the first letter and number (H18) corresponds to the amino acid histidine at position 18 in the unmodified hGH. The last letter corresponds to the amino acid which is substituted at the position (A for alanine).

4. The nomenclature for amino acids and polypeptide sequences is as follows:

Amino acid or residue	3-letter symbol	1-letter symbol
Alanine	Ala	A
Glutamate	Glu	E
Glutamine	Gln	Q
Aspartate	Asp	D
Asparagine	Asn	N
Leucine	Leu	L
Glycine	Gly	G
Lysine	Lys	K
Serine	Ser	S
Valine	Val	V
Arginine	Arg	R
Threonine	Thr	T
Proline	Pro	P
Isoleucine	Ile	I

Methionine	Met	M
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Cysteine	Cys	C
Tryptophan	Trp	W
Histidine	His	H

B. Modes of Carrying Out the Invention

1) Determination of Metal Ion Requirement

A determination of whether a metal ion is required for a given polypeptide hormone to bind to a given receptor may be made through the use of a physiological concentration of a given metal ion and a general di- and tri-valent metal chelating agent, such as EDTA, or a transition metal chelating agent such as 1,10 phenanthroline. Under conditions of 140 mM NaCl, 20 mM Tris (pH 7.5) at 25°C. (or similiar buffer conditions that mimic the salt and buffer concentrations in serum), the metal ion at levels ranging from 5 to 1000 μM, and below a concentration that would cause metal oxides or metal beffer complexes to precipitate is incubated with the polypeptide hormone (10-100pM) and the potential receptor (10-1000pM). The difference in the amount of polypeptide hormone binding to the receptor in the presence of the metal ion, and in the presence of an appropriate chelating agent, serves as an indicator of a metal ion requirement for receptor binding.

For example, to determine whether a zinc ion is required for hGH to bind to prolactin receptor, incubate hGH in the presence of zinc (50 μM), with either prolactin receptor or the binding region of prolactin receptor (10-100pM). The level of binding of the hGH to the prolactin receptor is determined. Next, in a second identical assay except for no added zinc, a chelating agent, such as EDTA, is added to the incubation mixture and the extent of hGH binding with the prolactin receptor determined by subtracting the value for plus EDTA from the value plus zinc. The difference in binding is a measure of the zinc ion requirement for prolactin receptor binding.

Metal ion specificity was determined and pharmacologic relevance of the Zn^{2+}·hGH·hPRLbp complex was analyzed. The total concentration of zinc in serum varies from 5 to 20 μM in the adult population (C. Lentner ed. in *Scientific Tables*, Eighth Ed., (Ciba-Geigy Ltd., Ardsley, N.Y., 1981), Vol. 3, pp. 79-88; R. Berfenstam, *Acta Paediat.* (Uppsala) 41, suppl 82 (1952)) and about 95 percent is complexed with proteins, mostly to serum albumin (Thorlacius-Ussing, *Neuroendocrinol.* **45**, 233 (1987); M. C. S. Koppelman, V. Greenwood, J. Sohn and P. Denster, *J. Clin. Endocrinol. Metab.* **68**, 215 (1989)). Thus, the free Zn^{2+} concentration in serum would be expected to range from about 0.1 to 5.0 μM or more preferably about 0.25 to 1 μM. This varies around the K_D (0.4 ± 0.2 μM) for Zn^{2+} binding to the hGH·hPRLbp

complex indicating that natural fluctuations in total zinc concentration can modulate the interaction between hGH and the hPRLbp complex.

It is nearly impossible to reconstitute serum so that the binding of specific metal ions can be tested independently under precisely physiological conditions because all metals compete to some extent for binding to serum proteins, notably serum albumin and metallothionene. Therefore, to translate our *in vitro* studies to a physiologically relevant setting is difficult. Nonetheless, in the presence of 0.1% (w/v) BSA (metal-free) the binding of hGH to the hPRLbp is modulated over a physiologically relevant range of total zinc (Fig. 2). Under these conditions we evaluated the ability of various divalent metal ions at physiologic concentrations to mediate the association between hGH and hPRLbp (Example 2, Table 5). At 60 pM hGH and hPRLbp, 20 μM Zn^{2+} supports about 50 percent complexation of hGH and hPRLbp whereas no other metals at their maximal expected total serum concentrations promote substantial binding. At much higher concentrations of hGH and hPRLbp (5 nM), zinc supports 75 percent complexation. This represents the maximum amount of complex that can be precipitated in our assay using a polyclonal antibody and polyethylene glycol. Other metals have no effect, except for Cu^{2+} and Ca^{2+} which support 29 percent complex formation. However, the dissociation constants (determined by Scatchard analysis) for binding of $[^{125}I]$hGH to hPRLbp in the presence of 5 mM $CaCl_2$ or 20 μM $CuSO_4$ are 21 (± 5 nM) and 11 (± 3) nM, respectively. These affinities are 300- to 600-fold weaker than for the zinc mediated complex (0.03 nM, Table 1). Thus, only zinc is capable of supporting strong binding between hGH and hPRLbp. However, other metal ions may function analogously in other polypeptide hormone-metal ion-receptor complexes.

Zinc plays a central role in many endocrine functions (A. S. Prasad, *Clin. Endocrinol. Metals* **14**, 567 (1985)) including growth hormone action. Zinc deficiency is often associated with alcoholism, pregnancy, some gastrointestinal disorders, severe burns, chronic renal failure, genetic disorders (acrodermatitis enteropathica and sickle cell anemia), and malnutrition. Moderate zinc deficiency leads to growth retardation (A. W. Root, G. Duckett, M. Sweetland, and E. O. Reiter, *J. Nutr.* **109**, 958 (1979); G. Oner, B. Bhaumick, and R. M. Bala, *Endocrinology* **114**, 1860 (1984); S. Kurtogu, T. E. Patiroglu and S. E. Karakas, *Tokai J. Exp. Clin. Med.* **12**, 325 (1987); Y. Nishi *et al.*, *J. Am. Coll. Nutr.* **8**, 93 (1989)) and hyperprolactinemia (M. C. Koppelman, *Med. Hypotheses* **25**, 65 (1988)). Our data provide a possible molecular basis for the association between zinc deficiency and altered growth hormone actions.

Zinc is a crucial component of the large class of zinc finger proteins (notably the steroid hormone receptors) that are important regulators of transcription (A. Klug and D. Rhodes, *Trends in Biochem. Sci.* **12**, 464 (1987); R. M. Evans and S. M. Hollenberg, *Cell* **52**, 1 (1988); J. M. Berg, *Cell* **57**, 1065 (1989)). Insulin is stored in complex with zinc in pancreatic cell secretory granules (J. C. Hutton, *Experientia* **40**, 1091 (1984); G.

Gold and G. M. Grodsky, *Experientia* **40**, 1105 (1984)). Our studies extend the involvement of zinc in hormone action by showing that it mediates directly the interaction between a polypeptide hormone and an extracellular receptor. The discovery that zinc promotes the binding between hGH and the hPRLbp depended critically on having highly purified component proteins free of contaminating cellular debris and metal ions. As more purified hormones and receptors become available, it is likely that zinc or other metal ions will be found to be directly involved in other polypeptide hormone-receptor interactions.

Any source of receptor may be used in the determination assay, including receptors bound to a cell surface, partially purified receptors or receptors produced by recombinant means. The formation of a polypeptide hormone-metal ion-receptor complex may be detected by commonly used assay procedures such as radionucleotides, enzyme immunoassays, or precipitation. The general methods as described in Example 1 may be adapted for determining metal ion requirements for any specific polypeptide hormone and receptor.

2) Determination of hGH Zinc Requirement

The expression of hPRLbp and the requirement for zinc ion was determined for hGH variants. To test many hGH variants for binding to the hGH receptor, we have relied upon an abundant and highly purified source of the extracellular binding domain (hGHbp) that was provided by an *E. coli* secretion system (G. Fuh *et al.*, *J. Biol. Chem.* **265**, 3111 (1990)). In a similar fashion, the extracellular domain of the hPRL receptor (hPRLbp) was expressed and secreted into the periplasm of *E. coli* (Fig. 1A). The hPRLbp was purified to near homogeneity from periplasmic extracts from *E. coli* by hGH affinity chromatography and gel filtration (Fig. 1B). The purified hPRLbp gave a single band of expected molecular weight (25 kD) on reduced SDS-PAGE. The purified hPRLbp had an amino-terminal sequence, Gln-Leu-Pro-Pro-Gly-Lys-Pro-Glu-Ile-Phe-Lys, indicative of proper cleavage of the signal peptide.

Initially, the binding of hGH to the purified hPRLbp was weak and highly variable compared to binding to unpurified hPRLbp from *E. coli* periplasm. A series of dialysis experiments, treatments with chelating agents, and divalent metal ions showed that zinc was required for tight binding of hGH to the hPRLbp. Titration with $ZnCl_2$ at a fixed concentration of [^{125}I]hGH and hPRLbp established that formation of the hormone-receptor complex was optimal at 50 µM $ZnCl_2$ (Fig. 2).

Under these conditions binding of hGH to hPRLbp is about 8,000-fold stronger in the presence of 50 µM $ZnCl_2$ compared to buffer containing 1 mM EDTA (Example 1, Table 1). In contrast, the binding constant of hPRL to the hPRLbp is essentially the same under either condition and is close to that measured previously for the full-length recombinant hPRL receptor (2-3 nM) (G. Fuh *et al.*, *J. Biol. Chem.* **265**, 3111 (1990)). Moreover, binding of hGH to hPRLbp in the presence of $ZnCl_2$ is nearly 100-fold

stronger than for hPRL, and more than 10-fold stronger than the affinity of hGH for the hGHbp. Scatchard analysis shows a stoichiometry of one hormone to one hPRLbp, and the binding of hGH is competitive with hPRL in the presence or absence of zinc, indicating that the hormone binding sites on the hPRLbp overlap. In contrast, zinc actually lowers the affinity of hGH to hGHbp by 4-fold, and prolactin does not bind to hGHbp in the presence or absence of $ZnCl_2$. Thus, the two receptors have fundamentally different metal ion requirements in addition to their well-known hormone specificities.

Interpretation of binding studies of hGH to receptors on cultured cells or tissues has often been problematic because usually these sources contain both prolactin and growth hormone (GH) receptors. Workers have traditionally used non-primate GH's or prolactins to differentiate these receptors. Our data suggest that binding of hGH in the presence of Zn^{2+} or EDTA can readily distinguish these two receptor classes. For example, $ZnCl_2$ enhances binding of hGH to rat adipocytes (A. C. Herington, *Biochem. Int.* **11**, 853 (1985)) which suggests these cells contain prolactin-like receptors. In addition, studies analyzing binding of hGH to prolactin receptors should be controlled for the presence of zinc. For instance, the IC_{50} value reported for binding of hGH to the recombinant full-length hPRL receptor in cell membranes is 0.26 nM (G. Fuh *et al., J. Biol. Chem.* **265**, 3111 (1990)) and to rat liver microsomes is 2-3 nM (J. Ray *et al., Mol. Endocrinol.* **4**, 101 (1990)) compared to the K_D value for the hPRLbp in the presence of 50 μM zinc of 0.03 nM. The discrepancies may reflect real differences in receptor affinities. However, earlier studies did not control the level of zinc in the binding assays.

Zinc binds to a single site at the interface between hGH and hPRLbp. Equilibrium dialysis experiments using $^{65}ZnCl_2$ were performed at a 1:1 ratio of hGH and hPRLbp (1.2 μM final) to determine the affinity and stoichiometry of zinc for the hGH·hPRLbp complex (Example 1, Fig. 3). Zinc binds in a non-cooperative fashion to a single site in the hGH·hPRLbp complex (the maximal level of bound Zn^{2+} is 1.0 μM in the presence of 1.2 μM hGH·hPRLbp complex) with an average K_D from this plus several independent experiments (not shown) of 0.4 (± 0.2) μM. Tight binding of zinc requires the presence of both hGH and hPRLbp, suggesting that the zinc site is at the interface of the complex.

Scanning-mutational analysis has identified about a dozen side-chains in hGH that mediate strongly the binding of hGH to hPRLbp in the presence of $ZnCl_2$ (Example 10). Of these residues, only the side-chains of His18, His21, and Glu174 are good candidate ligands for coordinating Zn^{2+}. These three residues are clustered (Example 1, Fig.4) when mapped upon a model of hGH built by homology to a folding diagram reported for porcine GH (S. S. Abdel-Meguid et al., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6434 (1987)). His18 and His21 are on adjacent turns of helix 1 and are positioned near

Glu174 on helix 4. All three face in the same direction and form a plausible site for binding of Zn^{2+}. In the presence of zinc, replacing either His18, His21 or Glu174 with alanine reduces the hormone affinity for the hPRLbp by about 100-fold relative to wild-type hGH (Example 1, Table.2). However, in the presence of 1 mM EDTA there is almost no difference in binding affinity between these mutants and wild-type hGH. Other alanine variants that disrupt binding of hGH to hPRLbp produce the same reduction in binding whether in the presence of zinc or EDTA. This indicates that the binding of zinc to the hGH·hPRLbp complex is mediated by the side-chains of His18, His21 and Glu174. The proximity of Asp171 to His18, His21 and Glu174 (Example 1, Fig.4) suggested it could also be a zinc ligand. However, the D171A mutation did not alter the binding of hGH and hPRLbp in the presence of zinc (Example 1, Table 2).

To further evaluate the direct involvement of His18, His21 and Glu174 in binding zinc with the hPRLbp, we analyzed the binding of low concentrations of $^{65}Zn^{2+}$ to each hGH mutant by equilibrium dialysis (Example 1, Table 3). The ratio of bound to free Zn^{2+} is dramatically reduced for these three mutants compared to wild-type hGH. The data show that the disruptive binding effects caused by alanine substitution of these residues correlate with disruption in zinc binding to the hGH·hPRLbp complex.

The model for the zinc binding site on hGH (Fig. 4) may account for the weak or undetectable binding of non-primate growth hormones to prolactin receptors. Sequence alignments (C. S. Nicoll, G. L. Mayer, S. M. Russel, *Endocrine Rev.* 7, 169 (1986)) of non-primate growth hormones show that all 19 non-primate GH's contain His21 and Glu174, but instead of His18 they contain Gln18. Interestingly, 17 of 19 non-primate GH's contain a histidine at position 19 (hGH contains Arg19). However, His19 can not coordinate zinc along with His21 because they are on opposite sides of helix 1 (Fig. 4). Of course, other differences between non-primate and primate GH's may contribute to the huge differences in binding affinities between these two subgroups of GH's for prolactin receptors.

Recently, it has been shown that a natural variant of hGH, known as hGH-V, binds more tightly to somatogenic than lactogenic receptors (J. Ray *et al., J. Biol. Chem.* **265**, 7939 (1990)). This homolog differs by only 13 residues out of 191 from hGH (P. H. Seeburg, *DNA* **1**, 239 (1982)). Remarkably, instead of His18 and His21, hGH-V contains Arg18 and Tyr21. Our studies suggest that hGH-V will not bind Zn^{2+} in association with the hPRL receptor, and that this is a major reason for its weaker binding.

The zinc binding site is positioned on the edge of the epitope identified for binding to the hGH receptor (Fig. 4). The model suggests zinc reduces binding of hGH to the hGHbp (Example 1, Table 1) by sterically interfering with binding of hGHbp. We have shown that binding to the hGHbp is enhanced about 4-fold by mutation of Glu174

to Ala . Thus, while Glu174 hinders binding of hGH to hGHbp, it is required for zinc mediated binding to hPRLbp.

Zinc typically coordinates four ligands in proteins (B. L. Vallee and A. Galdes, *Adv. Enzymol. Rel. Areas Molec. Biol.* **56**, 283 (1984); A. Klug and D. Rhodes, *Trends in Biochem. Sci.* **12**, 464 (1987); R. M. Evans and S. M. Hollenberg, *Cell* **52**, 1 (1988); J. M. Berg, *Cell* **57**, 1065 (1989)). Having identified three ligands from hGH and realizing that both hGH and the hPRLbp are required for tight zinc binding, we evaluated the possibility that the fourth ligand comes from the hPRLbp. We reasoned that the best candidates would be His or Cys residues which are conserved in hPRLbp but absent in the hGHbp. An alignment of four GH (W. C. Smith, J. Kuniyoshi, F. Talamantes, *Mol. Endocrinol.* **3**, 984 (1989); L. S. Mathews, B. Enberg, G. Norstedt, *J. Biol. Chem.* **264**, 9905 (1989)) and four PRL receptors sequences (J. M. Boutin *et al., Cell* **53**, 69 (1988); J. A. Davis, D. I. H. Linzer, *Mol. Endocrinol.* **3**, 674 (1989); M. Edery *et al., Proc. Natl. Acad. Sci. U.S.A.* **86**, 2112 (1989)) numbered according to the hPRLbp sequence (J. M. Boutin *et al., Mol. Endocrinol.* **3**, 1455 (1989)) shows that His159, Cys184, and His188 are completely conserved in all PRL receptors but not GH receptors.

We mutated His159, His188, and Cys184 separately to alanine and expressed them in *E. coli*. Of these three hPRLbp mutants, only H188A showed reduced binding affinity (Example 1, Table 4). Indeed, the binding affinity for hGH was reduced more than 2,000-fold for the H188A mutant in the presence of zinc. This was below the limit of accurate measurement in the assay. The mutational analysis supports a model wherein zinc is bound by three ligands from hGH (His 18, His21, Glu174) and one from hPRLbp (His 188).

3) <u>Structure of hPRL Receptor Binding Site On hGH</u>

To determine if the epitopes on hGH for the hGHbp and hPRLbp overlapped we analyzed whether the hPRLbp could displace the hGHbp from hGH. The hPRLbp competitively displaced the hGH binding protein from hGH (Fig. 6) suggesting their binding sites on the hormone overlap. The binding affinity of hGH to the hPRLbp is enhanced >8,000-fold in the presence of $ZnCl_2$ (Table 1). The dissociation constant for the hGH·hPRLbp complex measured here (68 pM) by competitive displacement of the hGHbp from hGH is roughly the same as that measured by direct binding of hGH to the hPRLbp under comparable concentrations of $ZnCl_2$ (25 μM). Higher concentrations of $ZnCl_2$ (50 μM) promote optimal binding between hGH and the hPRLbp (K_D = 38 pM; Table 10); however, such concentrations of $ZnCl_2$ reduce the affinity of hGH for the hGHbp by up to 4-fold.

Homolog-scanning mutagenesis (Cunningham, B. C., Jhurani, P., Ng, P. & Wells, J. A. *Science* **243**, 1330-1335 (1989)) was used to further localize the epitope on hGH for the hPRLbp (Table 10). In this approach, variants of hGH that contained

segment substitutions (7 to 30 residues long) derived from a non-binding homolog, pGH, or binding competent homologs, hPRL and hPL, were analyzed for binding to the hPRLbp. The hGH mutants containing segments of pGH, namely pGH (11-33) and pGH (57-73), cause large disruptions in binding affinity for the hPRLbp, whereas pGH (48-52) has no effect. As expected, virtually all of the substitutions tested from the binding competent hormones, hPRL and hPL, do not disrupt binding to hPRLbp. The only exception is hPRL (22-33) which causes a >15-fold reduction in binding affinity to the hPRLbp. Thus, binding to the hPRLbp is very sensitive to mutations in hGH near the central portion of helix 1 and the loop region between residues 57 and 73.

Several of the segment substituted variants cause substantial changes in receptor binding preference (Table 10) suggesting that the hPRL and hGH receptor epitopes are not identical. For example, the binding affinity of hPRL (22-33) or pGH (11-33) are 18 and 130-fold lower, respectively, for the hPRLbp than for the hGHbp. In contrast, the hPL (56-64) and hPRL (54-74) have almost no effect on binding to the hPRLbp, yet they weaken binding to the hGHbp by factors of 30 and 69, respectively. These preferential receptor binding effects for the hGH mutants coupled with their unaltered binding properties for a number of monoclonal antibodies indicate that reductions in receptor binding affinity are caused by local and not global structural changes in the variant hormones.

Next we identified specific side-chains in hGH that strongly modulate binding to the hPRLbp by alanine-scanning mutagenesis (Table 11). In addition to alanine-scanning the two regions implicated by the homolog-scanning to be involved in binding, we also scanned the helix 4 region because structurally this is in between the helix 1 and 54-74 loop region (Fig. 7). The alanine substitutions causing greater than a 4-fold reduction in binding affinity to the hPRLbp are in the central portion of helix 1 (including residues His18, His21, and Phe25), a loop region (including Ile58, Asn63, and Ser62) and the middle of helix 4 (comprising Arg167, Lys168, Lys172, Glu174, Phe176 and Arg178). These twelve residues form a patch when mapped upon a structural model of hGH (Fig. 7A). The most disruptive alanine substitutions in helix 1 and helix 4 project in the same direction. Three of these residues (His18, His21, and Glu174) along with His188 from the hPRLbp are believed to comprise the binding site for Zn^{2+} that is required for the high affinity hGH·hPRLbp complex.

The mutational analysis show that there are significant differences between the epitopes on hGH for the hGHbp and hPRLbp (Fig. 7). For example, with Zn^{2+} bound the net charge in the epitope on hGH for the hPRLbp is +5 (defined by residues causing ≥4-fold reduction in affinity (Fig 7A). This strongly electropositive charge cluster is surrounded by a series of important hydrophobic residues, Phe25, Ile58, Tyr164, and Phe176. Zinc is not required for formation of the hGH·hGHbp complex and the hGHbp epitope (Fig. 7B) is notably less electropositive (net charge for residues causing ≥4-fold

disruptions is +1). Overall, the hPRLbp epitope is elliptically shaped compared to the more circularly-shaped hGHbp.

Residues that cause large changes in receptor binding affinity may do so by indirect structural effects. We believe that the majority of these disruptive effects are due to local structural changes because virtually all of the single mutants tested retain full binding affinity to all of the hGH monoclonal antibodies. Moreover, the mutants often lead to changes in receptor binding preference and not disruptions in affinity for both receptors (Table 7).

Recently, it was shown by Ray et al.(*Mol. Endocrinol.* 4, 101-107 (1990)) that replacement of residues 32 to 71 or residues 72 to 125 in hGH with rat GH (rGH) or rat PRL (rPRL) sequences leads to alterations in binding to rat or rabbit liver microsomes which were used as crude sources of PRL and GH receptors, respectively. The disruptive effects they reported for replacement of residues 32-71 in hGH with rPRL or rGH are consistent with the data presented here. However, substitution of a 54-residue segment of hGH (residues 72 to 125) with rPRL causes disruptions of 10- to 1000-fold for both the GH and PRL receptors. They conclude that this sequence contains important binding determinants for GH receptors, but do not dissect them out, nor demonstrate the structural integrity of this mutant. Our data (Table 10) show that shorter hPRL sequence substitutions (residues 88 to 95, 97 to 104, and 111 to 129) in this region of hGH retain high binding affinity for both the highly purified hGH and hPRL binding proteins. Moreover, the structural integrities of the hGH mutants have been confirmed by monoclonal binding experiments. In addition, we have shown that the binding affinity for hPRL to the hGHbp can be enhanced to within 6-fold of hGH by installing only eight residues previously identified by alanine-scanning to be important for binding of hGH to hGHbp. None of these residues (S62, N63, E66, D171, E174, T175, F176, and R178) are between positions 72 and 125. While the 72 to 125 segment is likely to be critical for the overall structure of the hormone (it contains two of the 4 α-helical segments), our data do not support its direct involvement in hormone-receptor binding.

<u>**Design of receptor specific variants of hGH.**</u>

The high resolutional functional data show that the hGHbp and hPRLbp epitopes (Fig. 7A, 7B) overlap but do not superimpose. For example, Ile58, Lys172, and Phe176 are important for binding to either receptor (Fig. 7C). Other determinants are more important for binding to the hPRLbp (especially those involved in the Zn^{2+} site) and others for selective binding of hGHbp (notably Phe10, Glu56, Asp171, and Arg64). These data suggest that not all of the binding determinants for recognizing hGH are the same in the hGH and hPRL receptors.

If the changes in receptor binding free energy are additive, it should be possible to design highly specific variants of hGH by combining two receptor selective single alanine mutants. Indeed, when two single mutants that preferentially disrupt binding

hPRLbp (K168A and E174A) are combined, the double mutant exhibits a 35,000-fold shift in preference for binding to the hGHbp (Table 12). The preference for binding the hPRLbp over the hGHbp can be enhanced by nearly 150-fold by combining R64A and D171A. These receptor selective hGH variants (K168A, E174A or R64A, D171A) do not substantially reduce the affinity for the preferred receptor (hGHbp or hPRLbp, respectively). It is also possible to reduce binding to both receptors simultaneously. For example, combining K172A, and F176A, which individually cause large reductions in binding affinity to both receptors, produce much larger disruptions in affinity (500- to 8000-fold) than either of the two single mutants.

In all these instances the changes in the free energy of binding ($\Delta\Delta G_{binding}$) are strikingly additive (Table 13). Such simple additivity presents an extremely powerful strategy for engineering variants of hGH with desirable receptor binding affinity and specificity.

There are a number of similar cases to hGH where two or more receptors or receptor subtypes are known to exist, for instance the adrenergic receptors (Cascieri, M. A., Chicchi, G. G., Applebaum, J., Green, B. G., Hayes, N. S. & Bayne, M. L. *J. biol. Chem.* **264**, 2199-2202 (1989)); the IGF-I receptors (Cascieri, M. A., Chicchi, G. G., Applebaum, J., Green, B. G., Hayes, N. S. & Bayne, M. L. *J. biol. Chem.* **264**, 2199-2202 (1989)); IL-2 receptors (Robb, R. J., Greene, W. C. & Rusk, C. M. *J. Exp. Med.* **160**, 1126-1146 (1984); Robb, R. J. Rusk, C. M. & Neeper, M. P. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5654-5658 (1988)); and ANP receptors (Chang, M. S., Lowe, D. G., Lewis, M., Hellmiss, R., Chen, E., & Goeddel, D. V., *Nature* **341**, 68-72 (1989)). In these situations where a hormone exhibits broad receptor specificity it is difficult to link specific receptor function to a specific pharmacological effect. However, the use of receptor specific hormone analogs can greatly simplify this task; for example, catecholamine analogs were used to characterize β-adrenergic receptor subtypes and link specific receptor function to particular pharmacologic responses (Lefkowitz, R. J., Studel, J. M. & Caron, M. G. *A. Rev. Biochem.* **52**, 159-186 (1983)). By analogy, the receptor specific variants of hGH should be key reagents for probing the role of the hGH and hPRL receptors in the complex pharmacology of hGH, and for identifying other receptors for hGH.

Soluble Binding Proteins

Prolactin receptor binding protein lacking the transmembrane region was constructed as shown in Figure 5. Figure 5 shows a comparison of the mature hGHbp and the mature hPRLbp. Each of these binding proteins lacks the transmembrane region. Variants of the PRLbp may be constructed with additional polypeptide sequences at the amino or carboxy terminals. Human GHBP has been described and lacks an effective binding site for zinc ion. However, DNA encoding asparagine$_{218}$ can be mutated by conventional methods to create GHBP variants that has affinity for growth hormone

(Example 14). Using such conventional methods, the DNA encoding $asparagine_{218}$ was modified to encode alanine or histidine at position 218. The presence of $histidine_{218}$ resulted in a 30-fold increasing in affinity for hGH in the presence of zinc. Such complexes of hGH and GHBP incorporating zinc ion as a binding cofactor may be used as pharmaceutical formulations for therapeutic administration.

4) Variants with Metal Ion Binding Site Deleted or Inserted

Variants of polypeptide hormones or their receptors and binding proteins may be modified using site-directed mutagenesis or other well-known methods to modify amino acid residues to either delete required metal ion binding sites or to insert a metal ion binding site. The deletion of a metal ion binding site is exemplified by the modifications to human growth hormone and the modification to the soluble prolactin receptor protein. Similarly, the amino acid sequence of a polypeptide hormone or a hormone receptor not containing a metal ion binding site may be modified to create a variant that contains a metal ion binding site. Such a modification is exemplified by the modification to the human growth hormone binding protein by inserting $histidine_{218}$ in place of $arginine_{218}$, resulting in the formation of a hormone-receptor complex containing zinc ion.

The method of modifying a mammalian polypeptide hormone-receptor complex not containing a metal ion binding site to contain a metal ion binding site comprises determining the amino acid sequence of a polypeptide hormone or polypeptide hormone receptor not containing a metal ion binding site wherein the determined amino acid sequence has regions of homology with a known polypeptide hormone or receptor having a metal ion binding site. The amino acid sequence of the mammalian polypeptide hormone or hormone receptor not containing a metal ion binding site is modified to contain one or more amino acids analogous to the polypeptide hormone or hormone receptor containing a metal ion binding site. This results in the insertion of a metal ion binding site. The preferred metal ion binding site is zinc, although other metals may be used such as iron, nickel, copper, magnesium, manganese, cobalt, calcium and selenium. Selection methods for isolating the polypeptide hormone variants include any method that permits selection of those variants forming stable complexes with a metal ion, for example, phagemid isolation methods.

C. Methods

1) Antibody Production

To prepare antibodies, rabbits were immunized with *E. coli* derived hPRLbp using standard methods and serum was collected after each boosting. Serum was passed over an hGH column (7) in which the hPRLbp was covalently cross-linked to the hGH by reaction with dimethylsuberimidate (10 mM final in phosphate buffered saline; PBS). The hGH-hPRLbp column was washed sequentially with 2 M NaCl, 8 M urea, and 3 M KSCN to remove non-covalently bound components. Serum was passed over an hGH column to remove any anti-hGH antibodies that may have been produced from a low

level contamination by hGH during hGH affinity purification by the hPRLbp immunogen. The flow-through was adsorbed onto the hGH-hPRLbp column, washed with 1 M NaCl, and eluted with 3 M KSCN. The non-blocking anti-hPRLbp antibodies were dialyzed into PBS and titered for the optimal concentration needed to precipitate hPRLbp in the assay.

2) Cloning

In general, the DNA sequence encoding the parent polypeptide is cloned and manipulated so that is may be expressed in a convenient host. DNA encoding parent polypeptides can be obtained from a genomic library, from cDNA derived from mRNA, from cells expressing the parent polypeptide or by synthetically constructing the DNA sequence (Maniatis, T., et al. [1982] in Molecular Cloning, Cold Springs Harbor Laboratory, N. Y.).

The parent DNA is then inserted into an appropriate plasmid or vector which is used to transform a host cell. Prokaryotes are preferred for cloning and expressing DNA sequences to produce parent polypeptides, segment substituted to polypeptides variants. For example, E. coli K12 strain 294 (ATCC No. 31446) may be used as E. coli B, E. coli X1776 (ATCC No. 31537), and E. coli c600 and c600hfl, E. coli W3110 (F_-, γ_-, prototrophic, ATCC No. 27325), bacilli such as Bacillus subtilis and other enterobacteriaceae such as Salmonell typhimurium or Serratia marcesans, and various pseudomanas species. The preferred prokaryote is E. coli W3110 (ATCC No. 27325). When expressed in prokaryotes the polypeptides typically contain an N-terminal methionine or a formyl methionine, and are not glycosylated. These examples are, of course, intended to be illustrative rather than limiting.

In addition to prokaryotes, eukaryotic organisms such as yeast cultures or cells derived from multicellular organism may be used. In principle, any such cell culture is workable. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a repeatable procedure (Tissue Culture, Academic Press, Kruse and Patterson, editors [1973]). Examples of such useful host cell lines ar VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, W138, BHK, COS-7 and MDCK cell lines.

In general, plasmid vectors containing replication and co....ol sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as se :ences which encode proteins that are capable of providing phenotypic selection in transformed cells. For example, E. coli may be transformed using pBR322, a plasmid derived from an E. coli species (Mandel, M. et al. [1970] J. Mol. Biol. 53 , 154). Plasmid pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for selection. A preferred vector is pBO475 (Fig 8). This vector contains origins of

replication for phage and E. coli which allow it to be shuttled between such hosts thereby facilitating mutagenesis and expression.

"Expression vector" refers to DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribsome finding sites, and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

"Operably linked", when describing the relationship between two DNA or polypeptide regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

Once the parent polypeptide is cloned, site specific mutagenesis (Carter, P., et al. [1986] Nucl. Acids Res. 13, 6487), cassette mutagenesis (Wells, J. A., et al. [1985] Gene 34, 315), restriction selection mutagenesis (Wells, J. A., et al. [1986] Philos. Trans. R. Soc. London SerA 317, 415) or other known techniques may be performed on the cloned parent DNA to produce "segment-substituted DNA sequences" which encode for the changes in amino acid sequence defined by the analogous segment being substituted. When operably linked to an appropriate expression vector, segment-substituted polypeptides are obtained. In some case, recovery of the parent polypeptide or segment-modified polypeptide may be facilitated by expressing and secreting such molecules from the expression host by use of an appropriate signal sequence operably linked to the DNA sequence encoding the parent polypeptide or segment-modified polypeptide. Such methods are well-known to those skilled in the art. Of course, other methods may be employed to produce such polypeptides and segment-substituted polypeptides such as the in vitro chemical synthesis of the desired polypeptide (Banrany, G., et al. [1979] in The Peptides [eds. E. Gross and J. Meienhofer) Acad. Press, N. Y., Vol. 2, pp. 3-254).

The following examples are intended to illustrate the best mode now known for practicing the invention, but the invention is not to be considered limited to these examples.

EXAMPLE 1

ZINC REQUIREMENT FOR hGH BINDING TO hPRL RECEPTOR

Table 1 illustrates zinc dependence for binding of hGH or hPRL to their purified binding proteins (bp). Dissociation constants (K_D) for binding to hGHbp (0.1 nM final) were measured in assay buffer (20 mM Tris·HCl (pH 7.5), 0.1 percent w/v BSA), by competitive displacement of [^{125}I]hGH (2, 5-7). Binding to the hPRLbp (0.01 nM final) was measured in assay buffer containing 50 µM $ZnCl_2$ as described for Fig. 2. In the presence of $ZnCl_2$, the dissociation constants were identical whether analyzed by competition with [^{125}I]hGH or [^{125}I]hPRL; we report only those values determined by displacement of [^{125}I]hGH. For binding to the hPRLbp in the presence of 1 mM EDTA and no added divalent metal ions, [^{125}I]hPRL was the only suitable tracer because its binding affinity was essentially unaffected by EDTA.

Table 1

Receptor	K_D (± SD) nM hGH	K_D (± SD) nM hPRL
hPRLbp		
+$ZnCl_2$	0.033 (± 0.06)	2.6 (± 0.6)
+EDTA	270 (± 90)	2.1 (± 0.7)
hGHbp		
+$ZnCl_2$	1.6 (± 0.4)	>100,000
+EDTA	0.42 (± 0.07)	>100,000

Table 2 illustrates zinc dependence for binding of various alanine mutants of hGH to the hPRLbp. Dissociation constants between hGH and hPRLbp were determined as described for Fig. 2. Mutants of hGH were produced and purified as previously described. Variants are designated by the wild-type residue followed by the position in hGH and the mutant residue. WT = wild-type hGH; ND = not determined.

Table 2

hGH mutant	+$ZnCl_2$ (50 μM)		+EDTA (1 mM)	
	K_D (± SD) nM	$\frac{K_D(mut)}{K_D(WT)}$	K_D (± SD) nM	$\frac{K_D(mut)}{K_D(WT)}$
WT	0.033 ± 0.006	1	270 ± 90	1
H18A	4.5 ± 1.1	135	370 ±120	1.4
H21A	3.0 ± 1.0	91	200 ± 60	0.74
E174A	12.0 ± 3.0	356	360 ±120	1.3
D171A	0.037 ± 0.011	1.1	ND	–

Table 3 illustrates the ratio of bound to free $^{65}Zn^{2+}$ (0.2 μM total) at a fixed concentration of hGH mutant and hPRLbp (each 2 μM). $^{65}ZnCl_2$ was allowed to equilibrate in dialysis cells, and bound and free zinc concentrations were determined as described for Fig. 3.

Table 3

hGH mutant	[$^{65}Zn^{2+}$] bound/free
WT	7.2 ± 0.2
H18A	0.1 ± 0.1
H21A	1.6 ± 0.1
E174A	1.0 ± 0.2

Figure 2 illustrates the binding of [^{125}I]hGH to the hPRLbp in the presence of 0.1 percent BSA (crystallized high grade fraction V; Sigma), 140 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris (pH 7.5) and variable concentrations of total $ZnCl_2$. A fixed 1:1 ratio of hGH and hPRLbp (0.01 nM final) was incubated 16 h in the presence of the indicated concentration of $ZnCl_2$ and the bound [^{125}I]hGH was immunoprecipitated using affinity purified rabbit polyclonal antibodies directed against the hPRLbp. When the zinc concentration exceeded 100 μM, some protein precipitated, thus reducing the amount of native hGH·hPRLbp complex formed.

Figure 3 illustrates equilibrium dialysis for binding of $^{65}Zn^{2+}$ to the hGH·hPRLbp complex. All stock solutions were made from metal-free deionized water, and reagents were of highest quality available. Plastic dialysis cells and 3500 molecular weight cutoff membranes (preboiled in 5 percent w/w $NaHCO_3$ and washed) were

soaked in 1 mM EDTA and washed thoroughly with dialysis buffer containing 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$ (used to reduce non-specific binding of Zn^{2+}) dialysis membrane) and 140 nM NaCl. A 0.34 mM stock solution of $^{65}ZnCl_2$ (500 μCi/0.1 ml in 50 mM HCl; DuPont) was prepared from a 1 M $ZnCl_2$, 0.05 M HCl stock solution prepared gravimetrically from anhydrous $ZnCl_2$. One-half of the dialysis cell (0.2 ml total) contained hGH·hPRLbp (1.2 μM final) in dialysis buffer and the total zinc concentration was diluted over a range of 0.1 to 10 μM. $^{65}ZnCl_2$ was added initially on the side of the cell lacking hGH and the hPRLbp. Cells were sealed and rotated slowly for 16 hr at 25°C. Aliquots (50 μl) from each half of the dialysis cell were counted, and bound and free zinc concentrations were calculated. The binding studies were performed in the absence of carrier protein to avoid adventitious binding of Zn^{2+}.

Figure 4 illustrates the proposed Zn^{2+} binding site on hGH that mediates binding to the hPRLbp. Helical wheel projections show the amphipathic character of helix 1 and 4 with polar (shaded) and charged residues (blackened) on one face of the helix and non-polar (open) on the other. The positions of the putative zinc binding ligands, His18, His21, and Glu174, which are involved in binding hGH to the hPRLbp are shown (★). The region where hGH binds to the hGHbp is defined roughly by the shaded circle. Residues marked by the symbols •, ●, ⬤ and ○ represent sites where alanine mutations in hGH cause reductions of 2- to 4-fold, 4- to 10-fold, greater than 10-fold, or 4-fold increase in binding affinity for the hGHbp, respectively.

EXAMPLE 2

MUTATED hGH AND BINDING TO hPRL RECEPTORS

Table 4 illustrates the effect of mutations at conserved His and Cys residues in hPRLbp on binding of hGH in the presence of $ZnCl_2$. Mutants of the hPRLbp were produced by site-directed mutagenesis (25), purified and assayed in the presence of Zn^{2+} as described in Figs. 1, 2, and Table 1.

Table 4

hPRLbp mutant	K_D (± SD) nM
WT	0.050 ± 0.008
H159A	0.047 ± 0.008
C184A	0.045 ± 0.004
H188A	>100

EXAMPLE 3
EFFECT OF DIVALENT CATIONS

Table 5 illustrates the effect of divalent cations at physiological total serum concentrations (19) on the complexation of [^{125}I]hGH to hPRLbp (at 60 or 5,000 pM). Highly purified metal ion salts for $CaCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5\,H_2O$, $CoCl_2 \cdot 6\,H_2O$, $MnCl_2 \cdot 4\,H_2O$, $MgCl_2 \cdot 6\,H_2O$ were obtained from Johnson-Matthey Purotonic, Sigma, Mallinckrodt, Mallinckrodt and Johnson-Matthey Purotonic, respectively. Binding assays were performed as described in Fig. 2 in 0.1% BSA, 140 mM NaCl, 20 mM Tris (pH 7.5) at 25°C. The percentage of complex formed was calculated from the ratio of the amount of [^{125}I]hGH·hPRLbp complex immunoprecipitated to total [^{125}I]hGH present in the assay.

Table 5

Divalent metal	Concentration	Percent complex formation 60 pM	5000 pM
None	–	3.8 ± 1.4	5.4 ± 0.8
Ca^{2+}	5 mM	1.3 ± 0.7	29.0 ± 1.4
Co^{2+}	5 nM	1.3 ± 2.4	6.8 ± 1.5
Cu^{2+}	20 µM	1.7 ± 1.5	29.0 ± 1.4
Mg^{2+}	2 mM	0.8 ± 1.4	9.1 ± 3.6
Mn^{2+}	2 µM	0.4 ± 0.8	8.2 ± 1.8
Zn^{2+}	20 µM	46.0 ± 3.0	74.0 ± 1.0

EXAMPLE 4
EXPRESSION OF hPRLbp

Figure 1A illustrates the diagram of plasmid phPRLbp(1-211) which directs secretion of the hPRLbp into the periplasm of *E. coli*. The hPRLbp gene fragment is transcribed under control of the alkaline phosphatase (phoA) promoter and secreted under direction of the stII signal sequence. Genes are indicated by arrows, replication origins by circles, and restriction sites used in the construction are indicated. A cDNA encoding the hPRL receptor (3) in a Bluescript plasmid (Stratagene) was purchased from Dr. Paul Kelly (Royal Victorial Hospital, McGill University, Montreal, Canada). Site-directed mutagenesis (25) using an oligonucleotide with the sequence 5'–AGCCACAGAGATA<u>ACGCGT</u>CTATGTATCATTCAT-3' (Seq ID 4) was performed on this plasmid to introduce a stop codon and MluI restriction site (indicated by asterisks and underline, respectively) after the threonine 211 codon which

immediately precedes the transmembrane domain of the receptor. The 600 bp BglII-MluI fragment from this plasmid was then cloned into the NsiI-MluI backbone of plasmid phGHbp (1-246) (Boutin, J. M. *et al., Mol. Endocrinol.* 3, 1455 (1989)). A synthetic linker that spans the NsiI and BglII sites was used to fuse the hPRLbp onto the StII secretion signal sequence and restore the 5' end of the hPRLbp gene. The bottom strand of this linker has the sequence 5'–GATCTCAGGTTTTCCAGGA GGTAACTGTGCA–3' (Seq ID 5). The top strand is complementary to this but 4 bp shorter on each end to match the restriction site termini. Dideoxy sequencing (26) was used to confirm the construction.

Figure 1B illustrates Coomassie blue stained SDS-PAGE (12.5 percent) (U. K. Laemmli, *Nature* **227**, 680 (1970)) of purified hPRLbp. The hPRLbp was purified essentially as described for the hGHbp except that 50 μM $ZnCl_2$ was added to the ammonium sulfate precipitate prior to solubilizing and loading onto the hGH affinity column. The column was washed with 1 M KSCN and eluted with 2 M KSCN plus 50 mM NaCl, 0.02% NaN_3, 20 mM Tris·HCl (pH 7.5). The eluate was dialyzed into the same buffer minus KSCN and stored frozen (at -70°C). Lanes 1-5 are an *E. coli* periplasmic fraction, the $(NH_4)_2SO_4$ precipitate, the protein after hGH affinity chromatography, the wash just before elution of hPRLbp, and molecular weight standards (ranging from 14 to 97 kD), respectively.

EXAMPLE 5
HUMAN GROWTH HORMONE MUTAGENESIS AND EXPRESSION VECTOR

To facilitate efficient mutagenesis, a synthetic hGH gene was made that had 18 unique restriction sites evenly distributed without altering the hGH coding sequence. The synthetic hGH DNA sequence was assembled by ligation of seven synthetic DNA cassettes each roughly 60 base pairs (bp) long and sharing a 10 bp DNA fragment shown form NsiI to BglII. The ligated fragment was purified and excised from a polyacrylamide gel and cloned into a similarly cut recipient vector, pB0475, which contains the alkaline phosphatase promoter and stII signal sequence (Chang, C. N., et al. [1987] Gene 55, 189), the origin of replication for the phage f1 and pBR322 from bp 1205 through 4361 containing the plasmid origin of replication and the β lactamase gene. The sequence was confirmed by dideoxy sequence analysis (Sanger, F., et al. [1987] Proc. Natl. Acad. Sci. USA 74, 5463).

pBO475 was constructed as follows: the fI origin DNA from filamentous phage contained on a DraI, RsaI fragment 475bp in length was cloned into the unique PvuII site of pBr322 to make plasmid p652. Most of the tetracycline resistance gene was then deleted by restricting p652 with NheI and NarI, filling the cohesive ends in with DNA polymerase and dNTPs and ligating the large 3850bp fragment back upon itself to create the plasmid pΔ652. pΔ652 was restricted with EcoRI, EcoRV and the 3690bp fragment

was ligated for a 1300bp EcoRI, EcoRV fragment from phGH4R (Chang, C. N., et al [1987] Gene 55, 189) containing the alkaline phosphatase promoter, STII signal sequence and natural hGH gene. This construction was designated as pB0473. Synthetically derived DNA was cloned into pB0473, was restricted with NsiI, BglII, and ligated to a 420pb NsiI, HindIII fragment and a 1170bp hindII, BglII fragment, both derived from synthetic DNA. The resulting construction pB0475 contains DNA coding for the natural polypeptide sequence of hGH but possesses many new unique restriction sites to facilitate mutagenesis and further manipulation of the hGH gene. The entire DNA sequence of pB0475, together with the hGH amino acid sequence, is shown in Fig. 8. The unique restriction sites in hGH sequence in pBo475 allowed insertion of mutagenic cassettes (Wells, J. A., et al. [1985] Gene 34, 315) containing DNA sequences encoding analogous segments from the analogs pGH, hPL and hPRL.

The hGH and hGH variants were purified as follows: to 200g of cell paste, four volumes (800ml) of 10mM tris pH 8.0 was added. The mixture was place on an orbital shaker at room temperature until the pellets were thawed. The mixture was homogenized and stirred for an hour in a cold room. The mixture was centrifuged at 7000 for 15 min. The supernatant was decanted and ammonium sulfate was added to 45% saturation (277g/l) and stirred at room temperature for one hour. After centrifugation for 30 minutes at 11,000g, the pellet was resuspended in 40ml 10mM tris pH 8.0. This was dialyzed against 2 liters of 10mM tris pH 8.0 overnight. The sample was centrifuged or filtered over a 0.45 micron membrane. The sample was then loaded on a column containing 100ml of DEAE cellulose (Fast Flow, Pharmacia, Inc.). A gradient of from zero to 300mM NaCl IN 10mM tris H2Cl pH 8.0 overnight was run. Samples were concentrated to approximately 1mg/ml by Centri-Prep10 ultrafiltration.

EXAMPLE 6

EXPRESSION AND PURIFICATION OF SOLUBLE HUMAN GROWTH HORMONE RECEPTOR FROM E. COLI

Cloned DNA sequences encoding the soluble human growth hormone receptor hGHbp (Leung, D. W. et al. [1987] Nature 330, 537) were subcloned into expression vectors derived from pBO475.

E. coli W3110, degP (Strauch, K. L., et al. [1988] PNAS USA 85, 1576) was transformed with the expression vector and grown in low phosphate media (Chang, C. N. [1987] Gene 55, 189) in a fermenter at 30°C. The 246 amino acid hGHbp was used to generate preliminary data. A slightly shorter hGHbp containing amino acids 1 through 238 was used in the examples herein. The results obtained with that receptor were indistinguishable from those obtained with the 246 amino acid hGHbp.

EXAMPLE 7

RECRUITMENT OF BINDING PROTERTIES OF HUMAN GROWTH HORMONE INTO PLACENTAL LACTOGEN

Human placental lactogen (hPL) is reduced over 100-fold in binding affinity compared to hGH for hGH receptor (Baumann, G., et al. [1986] J. Clin. Endocrinol. Metab. 62, 134; Herington, A. C., et al. [1986] J. Clin. Invest. 77, 1817). Previous mutagenic studies showed the binding site on hGH for the hGH receptor is located primarily in two regions (including residues 54-74 and 171-185) with some minor determinants near the amino terminus (residues 1-14).

The overall sequence of hPL is 85% identical to hGH. Within the three regions that broadly constitute the receptor binding epitope on hGH, hPL differs at only seven positions and contains the following substitutions: P2Q, I4V, N12H, R16Q, E56D R64M, and I179M. (In this nomenclature the residues for wild-type hGH is given in single-letter code, followed by its position in mature hGH and then the residue found in hPL; a similar nomenclature is used to describe mutants of hGH.) Single alanine substitutions have been produced in hGH at each of these seven positions. Of these, four of the alanine substitutions were found to cause 2-fold or greater reduction in binding affinity including I4A, E56A, R64A and I179A. Generally, the alanine substitutions have a greater effect on binding than homologous substitutions from human prolactin. Therefore, the effect of some of the substitutions from hPL introduced into hGH were investigated. Whereas the I179A substitution caused a 2.7-fold reduction in affinity, the I179M caused only a slight 1.7-fold effect. However, the R64A and R64M substitutions caused identical and much larger reduction (about 20-fold) in binding affinity. Moreover, the double mutant (E56D:R64M) in hGH was even further reduced in affinity by a total of 30-fold. Thus, E56D and R64M primarily determine the differences in receptor binding affinity between hGH and hPL. The double mutant D56E, M64R in hPL therefore substantially enhances its binding affinity for the hGH receptor. Additional modifications such as M179I and V4I also enhance binding of hPL to the hGH receptor.

EXAMPLE 8

EFFECT OF AMINO ACID REPLACEMENT AT POSITION 174 ON BINDING TO THE HUMAN GROWTH HORMONE

As previously indicated, replacement of Glu174 with Ala (E174A) resulted in more that a 4-fold increase in affinity of human growth hormone (hGH) for its receptor. To determine the optimal replacement residue at position 174 hGH variants substituted with twelve other residues were made and measured to determine their affinities with the hGH binding protein (Table 6). Side-chain size, not charge, is the major factor determining binding affinity. Alanine is optimal replacement followed by Ser, Gly, Gln, Asn, Glu, His, Lys, Leu, and Tyr.

Table 6

	Side Chain			
Mutant	Charge	Size (A^3)[b]	Kd(nM)c	Kd(mut) / Kd(wild-type)
E174G	0	0	0.15	
E174A	0	26	0.075	0.22
E174S	0	33	0.11	0.30
E174D	-	59	NE	-
E174N	0	69	0.26	0.70
E174V	0	76	0.28	0.80
wild-type	-	89	0.37	1.0
E174Q	0	95	0.21	0.60
E174H	0	101	0.43	1.2
E174L	0	102	2.36	6.4
E174K	+	105	1.14	3.1
E174R	+	136	NE	-
E174Y	0	137	2.9	8.6

a) Mutations were generated by site-directed mutagenesis (Carter, P., et al. [1986] Nucleic Acid Res. 13, 4431-4443) on variant of the hGH gene that contains a KpnI site at position 178 cloned into pB0475. Oligonucleotides used for mutagenesis had the sequence:

* * *

5'-AC-AAG-CTC-NNN-ACA-TTC-CTG-CGC-ATC-GTG-CAG-T-3' (Seq ID 6)

where NNN represents the new codon at position 174 and asterisks indicate the mismatches to eliminate the KpnI site starting at codon 178. Mutant codons were as follows: Gln, CAG; Asn, AAC; Ser, AGC; Lys, AAA; Arg, AGG; His, CAC; Gly, GGG ; Val, GTG; GTG; Leu, CTG. Following heteroduplex synthesis, the plasmid pool was enriched for the mutation by restriction with KpnI to reduce the background of wild-type sequence. All mutant sequences were confirmed by dideoxy sequence analysis (Sanger, F., et al. [1977] Proc. Natl. Acad. Sci. USA 74, 5463-5467).

b) Side-chain packing values are from Chothia, C., (1984) Annu. Rev. Biochem. 53, 537.

c) Dissociation constants were measured by competitive displacement of [125I]hGH from the hGH binding protein as previously described. NE indicates that the mutant hormone was expressed as levels too low to be isolated and assayed.

EXAMPLE 9

RELATIONSHIP BETWEEN HUMAN GROWTH HORMONE AND HUMAN PROLACTIN RECEPTOR BINDING SITES

Table 7 illustrates the comparative binding of hGH variants to the hPRLbp and hGHbp. Mutants of hGH produced by homolog-scanning mutagenesis are named according to the extremes of the segment substituted from the various hGH homologs: pGH, hPL, or hPRL. The exact description of the mutations introduced is given by the series of single mutants separated by commas. The component single mutants are designated by the single-letter code for the wild-type residue followed by its codon position in mature hGH and then the mutant residue. Mutants of hGH were produced and purified as previously described. Binding of the hGH mutants to the hPRLbp was measured by competitive displacement of [^{125}I]hGH as described for the hGHbp except that assays included 50 μM $ZnCl_2$ and 10 mM $MgCl_2$. Affinity purified rabbit polyclonal antibodies raised against the hPRLbp were used to precipitate the hGH•hPRLbp complex. The relative reduction in binding affinity (K_D(mut)/K_D(hGH)) reported for the hGHbp was taken from S. S. Abdel-Meguid et al., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6434 (1987). The change in receptor preference was calculated by dividing the ratio of the relative reduction in binding affinity for the hPRLbp by that for the hGHbp. WT = wild-type; SD = standard deviation.

We identified specific side-chains in hGH that strongly modulate binding to the hPRLbp by alanine-scanning mutagenesis (Table 8). In addition to alanine-scanning the two regions implicated by the homolog-scanning to be involved in binding, we also scanned the helix 4 region as structurally this is in between the helix 1 and 54-74 loop region. The alanine substitutions causing greater than a 4-fold reduction in binding affinity to the hPRLbp are in the central portion of helix 1 (including residues His18, His21, and Phe25), a loop region (including Ile58, Asn63, and Ser62) and the middle of helix 4 (comprising Arg167, Lys168, Lys172, Glu174, Phe176 and Arg178). These twelve residues form a patch when mapped upon a structural model of hGH (Fig.7A). The most disruptive alanine substitutions in helix 1 and helix 4 project in the same direction. Three of these residues, (His18, His21, and Glu174) along with His188 from the hPRLbp, are believed to comprise the binding site for Zn^{2+} that is required for the high affinity hGH•hPRLbp complex.

Table 7

Mutant name	Mutations introduced	hPRLbp K_D (pM) ± SD	hPRLbp $\frac{K_D(\text{mut})}{K_D(\text{hGH})}$	hGHbp $\frac{K_D(\text{mut})}{K_D(\text{hGH})}$	Change in preference for binding the hGHbp over over hPRLbp
WT hGH	none	33 ± 3	(1)	(1)	(1)
pGH (11-33)	D11A, M14V, H18Q, R19H, F25A, Q29K, E33R	14,500 ± 300	440	3.4	130
pGH (48-52)	P48A, T50A, S51A, L52F	50 ± 10	1.5	2.8	0.54
pGH (57-73)	S57T, T60A, S62T, N63G, R64K, E65D, T67A, K70R, N72D, L73V	1,600 ± 350	49	17	2.9
hGH (Δ32-46)	Deletion of residues 32 to 46	200	6.1	ND	-
hPL (46-52)	Q46H, N47D, P48S, Q49E, L52F	50 ± 13	1.5	7.2	0.21
hPL (56-64)	E56D, R64M	70 ± 14	2.1	30	0.07
hPRL (12-19)	N12R, M14V, L15V, R16L, R19Y	63 ± 14	1.9	17	0.11
hPRL (22-33)	Q22N, F25S, D26E, Q29S, E30Q, E33K	500 ± 155	15	0.85	18
hPRL (54-74)	F54H, S55T, E56S, I58L, P59A S62E, N63D, R64K, E66Q, T67A K70M, S71N, N72Q, L73K, E74D	60 ± 18	1.8	69	0.03
hPRL (88-95)	E88G, Q91Y, F92H, R94T, S95E	47 ± 12	1.4	1.4	1.0
hPRL (97-104)	F97R, A98G, N99M, S100Q, L101D, V102A, Y103P, G104E	63 ± 8	1.9	1.6	1.2
hPRL(111-129)	Y111V, L113I, K115E, D116Q, E118K, E119R, G120L, Q122E, T123G, G126L, R127I, E129S	17 ± 5	0.5	1.5	0.33

Table 8

Mutant	hPRLbp K_D (pM) ± SD	hPRLbp $\frac{K_D(\text{mut})}{K_D(\text{hGH})}$	hGHbp $\frac{K_D(\text{mut})}{K_D(\text{hGH})}$	Change in receptor preference $\frac{\text{hGHbp}}{\text{hPRLbp}}$
WT hGH	33 ± 6	1.0	1.0	1
Δ(1-8)	93 ± 26	2.8	ND	–
F10A	33 ± 12	1.0	5.9	0.17
N12A	20 ± 6	0.6	1.2	0.5
L15A	33 ± 7	1.0	1.3	0.77
R16A	33 ± 6	1.0	1.5	0.67
H18A	4,500 ± 1,100	140	1.6	88
R19A	33 ± 10	1.0	0.7	1.4
H21A	3,000 ± 1,000	91	ND	–
F25A	230 ± 50	7	ND	–
D26E	63 ± 11	1.9	ND	–
F54A	47 ± 8	1.4	4.4	0.32
S55A	50 ± 15	1.5	1.2	1.3
E56A	27 ± 5	0.8	4.1	0.20
S57A	35 ± 11	1.0	1.4	0.71
I58A	611 ± 110	18	17	1.1
P59A	43 ± 22	1.3	1.9	0.68
S62A	360 ± 100	11	2.8	3.9
N63A	140 ± 46	4.3	3.3	1.3
R64A	60 ± 15	1.8	21	0.09
E65A	83 ± 29	2.5	0.59	4.2
E66A	37 ± 6	1.1	2.1	0.52
Q68A	40 ± 6	1.2	5.2	0.23
Q69A	23 ± 8	0.7	0.91	0.77
K70A	50 ± 15	1.5	2.4	0.63
S71A	93 ± 20	2.8	2.0	1.4
L73A	60 ± 22	1.8	0.71	2.5
Y160A	47 ± 8	1.4	1.1	1.3
Y164A	70 ± 13	2.1	3.6	0.58
R167A	25,000 ± 5,000	770	0.75	1,030
K168A	610 ± 120	18	1.1	16

D171A	37 ± 11	1.1	7.1	0.15
K172A	7,200 ± 1,100	220	14	16
E174A	12,000 ± 1,700	356	0.22	1,600
T175S	76 ± 15	2.3	3.5	0.66
F176A	830 ± 100	25.0	16.0	1.6
R178A	230 ± 20	7.0	ND	
I179A	60 ± 5	1.8	2.7	0.67
I179M	25 ± 2	0.75	2.7	0.28
V180A	20 ± 2	0.6	1.0	0.60
Q181A	33 ± 4	1.0	1.6	0.63
R183A	86 ± 6	2.6	2.1	1.2
S184A	27 ± 2	0.8	0.91	0.88
V185A	53 ± 5	1.6	4.5	0.36
E186A	33 ± 6	1.0	0.79	1.3
G187A	33 ± 3	1.0	1.8	0.56
S188A	20 ± 2	0.6	0.71	0.85

Table 9 illustrates the binding of double mutants of hGH designed to discriminate between the hGH and hPRL binding proteins (hGHbp and hPRLbp). Mutants of hGH were prepared by site-directed mutagenesis, purified (Cunningham, B. C. & Wells, J. A. *Science* **244**, 1081-1085 (1989)), and assayed for binding to the hGHbp (Fuh, G., Mulkerrin, M. G., Bass, S., McFarland, N., Brochier, M., Bourell, J. H., Light, D. R., & Wells, J.A. *J. Biol. Chem.* **265**, 3111-3115 (1990)) or hPRLbp as described in Table 7.

Table 9

	hPRLbp		hGHbp		Change in receptor preference
Mutant $\frac{K_D(\text{mut})}{K_D(\text{hGH})}$	K_D (pM) $\frac{\text{hGHbp}}{\text{hPRLbp}}$	$\frac{K_D(\text{mut})}{K_D(\text{hGH})}$		K_D (pM)	
WT hGH	38 ± 3	(1)	440 ± 40	(1)	(1)
K168A, E174A	350,000 ± 80,000	9,100	120 ± 10	0.27	34,000
R64A, D171A	72 ± 7	1.9	120,000 ± 15,000	280	0.0068
K172A, F176A	320,000 ± 70,000	8,400	260,000 ± 130,000	560	15

Table 10 illustrates the additive effects of mutations in hGH upon binding to the hGH or hPRL binding proteins. The change in the free energy of binding ($\Delta\Delta G_{binding}$) for the variant relative to wild-type hGH was calculated from the reduction in binding affinity according to: $\Delta\Delta G_{binding} = RT \ln\left[\frac{K_D(\text{mut})}{K_D(\text{hGH})}\right]$. The values of ($K_D$(mut)/$K_D$(hGH)) for the single or multiple mutant hormones were taken from Tables 8-11.

Table 10

Mutation	Change in binding free energy, $\Delta\Delta G_{binding}$ (kcal/mol) hGHbp	hPRLbp
K168A	+0.1	+1.7
E174A	-0.9	+3.5
K168A, E174A (expected)	-0.8	+5.2
(actual)	-0.8	+5.4
R64A	+1.8	+0.3
D171A	+1.2	+0.1
R64A, D171 (expected)	+3.0	+0.4
(actual)	+3.4	+0.4
K172A	+1.6	+3.2
F176A	+1.7	+1.9
K172A, F176A (expected)	+3.3	+5.1
(actual)	+3.8	+5.4

Figure 6 illustrates the competition between hGH and hPRL binding proteins for binding to [^{125}I]hGH. The concentrations of [^{125}I]hGH and purified hGHbp domain were fixed at 0.2 nM. Increasing concentrations of purified hPRLbp were added and the three components were allowed to reach equilibrium in assay buffer containing 25 µM $ZnCl_2$, 20 mM Tris·HCl (pH 7.5) and 0.1 percent w/v BSA for 12 h at 25°C. A non-neutralizing monoclonal antibody to the hGHbp (Mab263, Bernard, R., Bundesen, P. G., Rylatt, D. B., & Waters, M. J. *Endocrinology* **115**, 1805-1813 (1984)) was added to precipitate the hGHbp with any [^{125}I]hGH that remained bound to it as previously described. The inset plot shows the data reformulated in a Scatchard plot to calculate of the K_D (68 pM) between hGH and the hPRL bp.

Figure 7 illustrates the structural model of hGH based on a folding diagram for pGH determined from a 2.8 Å resolution X-ray structure (Abdel-Megnid, S. S., Shieh,

H. S., Smith, W. W., Dayringer, H. E., Violand, B. N., & Bentle, L. A. *Proc. Natn. Acad. Sci. U.S.A.* **84**, 6434-6437 (1987)). Panel A shows a functional map of the hPRLbp epitope, and Panel B shows that determined previously for the hGH bp (taken from S. S. Abdel-Meguid et al., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6434 (1987)). The symbols •, ●, ● and ● represent sites where alanine substitutions cause a 2- to 4-fold, 4- to 10-fold, 10-fold to 80-fold, or >80-fold reductions in binding affinity, respectively, for each receptor binding domain. The ○ in the hGHbp epitope (Panel B) represents the position of E174A that causes greater than a 4-fold increase in binding affinity. Panel C shows sites where alanine mutants reduce binding affinity by ≥10-fold for hPRLbp (□) or ≥5-fold for the hGHbp (■) without affecting substantially the binding to the hGHbp or hPRLbp, respectively. The ▲ symbols show sites where alanine mutants disrupt binding to both receptors by ≥10-fold.

EXAMPLE 11

ZINC DEPENDENT BINDING OF HUMAN GROWTH HORMONE TO THE HUMAN PROLACTIN RECEPTOR.

The binding of human growth hormone (hGH) to human prolactin (hPRL) receptor has been shown here to be zinc-dependent (Table 11). The data were normalized to the expected Kd for the labelled ligand. Zinc-containing binding assays were performed in the presence of 1mM EDTA (without zinc), or 10 mM $MgCl_2$ and 50 μM $ZnCl_2$ (plus zinc) using displacement of labelled hGH from the prolactin receptor (Table 12). An unlabelled hGH competition assay was included with each experiment and used to standardize the Kd values. Zinc-free binding assays were performed in the absence of added Mg^{++} or Zn^{++} and in the presence of 1 mM EDTA using displacement of labelled hPRL from the prolactin receptor.

Table 11

Kd For Hormone Binding to hPRL Receptor

HORMONE	PLUS ZINC	WITHOUT ZINC
hPRL	2.6 nM	2.8 nM
hGH	33 pM	270 nM
hPL	50 pM	hPRL PPTS.

Plus zinc contained 50 μM $ZnCl_2$ and 10 mM $MgCl_2$ and minus zinc contained 1 mM EDTA, no added zinc or magnesium. At higher hPL concentrations the hPL and hPRL aggregate with the prolactin receptor to form a precipitate. In the presence of 1 mM EDTA the absence of zinc causes formation of a PEG-precipitate of hPL in assays using labelled hPRL. Half-maximal precipitation of hPRL occurred at 240 nM hPL.

This may be of physiological significance because maternal serum concentrations of hPL approach 300 nM. In addition, prolactin receptor can compete with hPL for binding to the prolactin. The binding of hPL to the prolactin receptor has a Kd of greater than 10 nM.

Table 12 shows the binding of hPL mutants to the hPRL receptor in the presence of 10 mM $MgCl_2$ plus 50 μM zinc using displacement of labelled hGH from the prolactin receptor.

Table 12
Binding hPL Mutants to hPRLbp

hPL Mutant	Kd (pM)	Kd(mut)/Kd(hGH)
hGH	33	1.0
hPL	50	1.5
D56E	27	0.8
M64R	34	1.0
E174A	>9000	>270
M179I	33	1.0
D56E,M64R,M179I	27	0.8
V4I,D56E,M64R,M179I	48	1.5

Kd (nM) values for mutants of hPL were determined using recombinant human growth hormone binding protein (hGHbp) (Table 13).

Table 13
hGHbp Binding Analysis of hPL Mutants

hPL Mutant	Kd(nM)	S.D.%	Kd(mut)/Kd(hGH)
1. hPL	949.20	11.55	2260.00
2. V4I	446.88	55.80	1062.00
3. E174A	309.12	20.60	736.00
4. HGH(65-191)	279.30	35.71	665.00
5. M179I	189.42	44.21	451.00
6. D56E	143.64	43.75	342.00
7. hGH(25-64)	62.58	13.00	149.00
8. M64R	42.84	30.23	102.00
9. D56E, M179I	20.20	15.00	48.10
10. M64R,N179I	13.90	22.50	33.10
11. D56E,N64R,M179I	6.01	24.20	14.30
12. #13 + T34A,H153D	5.54	17.80	13.20
13. V4I,D56E,M64R,M179I	4.70	14.70	11.20
14. #13 + H47Q,S48P	2.20	28.50	11.00
15. D56E,M64R,E174A,M179I	2.53	31.05	6.02
16.V4I,D56E,M64R,E174A,M179	1.72	17.07	4.10
17. hGH(25-191)	1.11	20.49	2.64
18. hGH	1.00	14.01	1.00

EXAMPLE 14

A VARIANT HUMAN GROWTH HORMONE RECEPTOR WHICH BINDS HUMAN GROWTH HORMONE IN A ZINC-DEPENDENT MANNER

Studies of the interaction between human growth hormone (hGH) and the extracellular domain of human prolactin receptor (hPRLr) have shown that the binding affinity is increased about 8000-fold in the presence of 50µM $ZnCl_2$ (B. C. Cunningham *et al.* [1990], Science 250 pp. 1709-1712). These studies demonstrated further that the interaction with zinc was mediated by amino acid residues His18, His21 and Glu174 in the growth hormone and His188 in the prolactin receptor.

Table 14 shows a comparison of part of the amino acid sequence of growth hormone and prolactin receptors from several different species. The histidine at position 188 in the prolactin receptors is conserved; furthermore, no histidine is present in any of the growth hormone receptors at the corresponding position (residue 218). Site-directed mutagenesis of residue 188 in hPRLbp has demonstrated that it is essential for the high affinity zinc-mediated binding of hGH (Example 1, Table 4).

Table 14
Comparison of GH andProlactin Receptor Sequences from Various Sepcies

human GHr	VRVRSKQRNSGNYGEFSE	(Seq ID 7)
rabbit GHr	VRVRSRQRSSEKYGEFSE	(Seq ID 8)
mouse GHr	VRVRSRQRSFEKYSEFSE	(Seq ID 9)
rat GHr	VRVRSRQRSFEKYSEFSE	(Seq ID 10)
human PRLr	VQVRCKPDHGYWSAWSPA	(Seq ID 11)
rabbit PRLr	VQVRCKPDHGFWSVWSPE	(Seq ID 12)
mouse PRLr	VQTRCKPDHGYWSRWGQQ	(Seq ID 13)
rat PRLr	VQTRCKPDHGYWSRWSQE	(Seq ID 14)
	*	

* indicates residue 188 in hPRLr sequence, 218 in hGHr sequence

In an attempt to recruit zinc-mediated binding into the hGH:hGHbp interaction, site-directed mutagenesis was carried out on residue Asn218 in hGHbp. Two mutant genes were constructed, coding for hGHbp mutants N218H and N218A. It was hoped that introducing a histidine at position 218 would enable the mutant hGHbp to interact with hGH via a Zn^{2+} ligand as in the case of the hGH:hPRLbp interaction. The N218A mutant was generated as a "neutral" control to investigate the effect of deleting the asparagine without introducing any novel functional group. The hGHbp expression vector has been described previously (G. Fuh *et al.* [1990], J. Biol. Chem. 265 pp. 3111-3115) and is similar to the hPRLbp expression vector of Fig. 1, with the hGHbp gene in place of the hPRLbp gene. Site-directed mutagenesis was performed using the method of Kunkel (T. A. Kunkel *et al*. [1987], Methods Enzymol. 154, 367-382); the mutagenic oligonucleotides used were:

5'-TCCAAACAACGACACTCTGGAAATTAT-3' for N218H (Seq ID 15)

5'-TCCAAACAACGAGCCTCTGGAAATTAT-3' for N218A (Seq ID 16)

The mutant binding proteins were expressed in E. Coli KS330 cells and either partially purified by fractionation with 45% ammonium sulphate or extensively purified using an hGH affinity column. Binding of the mutant receptors to hGH in the presence of 50μM $ZnCl_2$ or 1mM EDTA was measured by competitive displacement of [^{125}I] hGH by unlabelled hGH as previously described (S. A. Spencer *et al.* [1988], J. Biol. Chem. 263 pp. 7862-7867). The amount of wild-type or mutant binding protein used in the assay was determined empirically by titration of the binding protein with [^{125}I] hGH: the concentration of binding protein in the assay was chosen to be that which gave approximately 20% [^{125}I] hGH bound in the preliminary titration.

Table 15
K_D values for the interaction of wild-type and mutant hGHbp in the presence or absence of 50μM $ZnCl_2$

VARIANT	MINUS $ZnCl_2$	PLUS $ZnCl_2$
WT	0.42±0.07	1.6±0.4
N218A	1.04±0.13	0.25±0.07
N218H	0.58±0.06	0.047±0.009

(All K_D values are in units of nM)

Table 15 shows the effect of 50μM $ZnCl_2$ on binding of hGH to wild-type hGHbp and to hGHbp mutants N218H and N218A. With the N218H mutant, the binding is not significantly different to wild-type in the absence of zinc, but in the presence of 50μM $ZnCl_2$ the binding is dramatically (>30-fold) tighter, presumably due to the incorporation of a zinc ligand into the interaction. The N218A mutant shows the effect of removing the asparagine side-chain without introducing a zinc ligand: binding to growth hormone is approximately 2-fold weaker than wild-type in the absence of Zn^{2+} and 6-fold tighter in the presence of Zn^{2+}. This indicates that, in the presence of zinc, replacing asparagine$_{218}$ with alanine enhances the binding by relieving a bad intermolecular contact whereas the tight binding of the histidine$_{218}$ region in the variant is due to the introduction of a new interaction between the inserted histidine and zinc. The presence of such tight binding in the complex between growth hormone, growth hormone binding protein and zinc facilitates the preparation of stable formulations. Similarly, the administration of growth hormone binding protein containing histidine$_{218}$ in the presence of zinc will facilitate the formation of stable complexes with endogenous growth hormone.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.
Bass, Steven H.
Cunningham, Brian C.
Fuh, Germaine
Lowman, Henry B.
Matthews, David J.
Wells, James A.

(ii) TITLE OF INVENTION: Metal Ion Mediated Receptor Binding Of Polypeptide Hormones

(iii) NUMBER OF SEQUENCES: 16

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: patin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 16-Aug-1991
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 07/568936
(B) APPLICATION DATE: 17-Aug-1990

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Benson, Robert H.
(B) REGISTRATION NUMBER: 30,446
(C) REFERENCE/DOCKET NUMBER: 655p1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/266-1489
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Phe Ser Cys His Trp Thr Asp Glu Val His His Gly Thr Lys Asn
                 50                  55                  60

Leu Gly Pro Ile Gln Leu Phe Tyr Thr Arg Arg Asn Thr Gln Glu
                 65                  70                  75

Trp Thr Gln Glu Trp Lys Glu Cys Pro Asp Tyr Val Ser Ala Gly
                 80                  85                  90

Glu Asn Ser Cys Tyr Phe Asn Ser Ser Phe Thr Ser Ile Trp Ile
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Pro Tyr Cys Ile Lys Leu Thr Ser Asn Gly Gly Thr Val Asp Glu
                110                 115                 120

Lys Cys Phe Ser Val Asp Glu Ile Val Gln Pro Asp Pro Pro Ile
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Ala Leu Asn Trp Thr Leu Leu Asn Val Ser Leu Thr Gly Ile His
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                155                 160                 165

Gln Lys Gly Trp Met Val Leu Glu Tyr Glu Leu Gln Tyr Lys Glu
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 211 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Thr Leu Met His Glu Cys Pro Asp Tyr Ile Thr Gly Gly Pro Asn
                50                  55                  60

Ser Cys His Phe Gly Lys Gln Tyr Thr Ser Met Trp Arg Thr Tyr
                65                  70                  75

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                140                 145                 150

Glu Lys Ala Ala Glu Trp Glu Ile His Phe Ala Gly Gln Gln Thr
                155                 160                 165

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                170                 175                 180

Gln Val Arg Cys Lys Pro Asp His Gly Tyr Trp Ser Ala Trp Ser
                185                 190                 195

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Thr
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4916 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AAAAGTTAAT CTTTTCAACA GCTGTCATAA AGTTGTCACG GCCGAGACTT 350

ATAGTCGCTT TGTTTTTATT TTTTAATGTA TTTGTAACTA GTACGCAAGT 400

TCACGTAAAA AGGGTATCTA GAGGTTGAGG TGATTTT    ATG AAA 443
                                            Met Lys
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120                 125                 130

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Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln
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185                 190                 195

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TTATCATGAC ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT 4910

CTTCAA 4916

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCCACAGAG ATAACGCGTC TATGTATCAT TCAT 34

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCTCAGGT TTTCCAGGAG GTAACTGTGC A 31

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACAAGCTCNN NACATTCCTG CGCATCGTGC AGT 33

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Val Arg Val Arg Ser Lys Gln Arg Asn Ser Gly Asn Tyr Gly Glu
 1               5                  10                  15

Phe Ser Glu
        18
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Arg Val Arg Ser Arg Gln Arg Ser Ser Glu Lys Tyr Gly Glu Phe
 1               5                  10                  15

Ser Glu
    17
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Val Arg Val Arg Ser Arg Gln Arg Ser Phe Glu Lys Tyr Ser Glu
 1               5                  10                  15

Phe Ser Glu
        18
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Val Arg Val Arg Ser Arg Gln Arg Ser Phe Glu Lys Tyr Ser Glu
 1               5                  10                  15

Phe Ser Glu
        18
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Val Gln Val Arg Cys Lys Pro Asp His Gly Tyr Trp Ser Ala Trp
 1               5                  10                  15

Ser Pro Ala
        18
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Val Gln Val Arg Cys Lys Pro Asp His Gly Phe Trp Ser Val Trp
 1               5                  10                  15

Ser Pro Glu
        18
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Val Gln Thr Arg Cys Lys Pro Asp His Gly Tyr Trp Ser Arg Trp
 1               5                  10                  15

Gly Gln Gln
        18
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Val Gln Thr Arg Cys Lys Pro Asp His Gly Tyr Trp Ser Arg Trp
 1               5                  10                  15

Ser Gln Glu
        18
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCCAAACAAC GACACTCTGG AAATTAT 27

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCCAAACAAC GAGCCTCTGG AAATTAT 27

WHAT IS CLAIMED IS:

1. A method of modifying a mammalian polypeptide hormone-receptor complex containing a metal ion binding site wherein the presence of a metal ion in said metal ion binding site determines the hormone's affinity for the mammalian hormone receptor comprising replacing a histidine, glutamate, aspartate or cysteine amino acid in a mammalian polypeptide hormone or receptor that chelates said metal ion to said mammalian polypeptide hormone-receptor complex, with another amino acid to prepare a variant hormone or receptor that is reduced in its ability to chelate said metal ion.

2. The method of claim 1 wherein said metal ion is selected from the group consisting of zinc, iron, nickel, copper, magnesium, manganese, cobalt, calcium or selenium.

3. The method of claim 2 wherein said metal ion is zinc.

4. The method of claim 3 wherein said mammalian polypeptide hormone is growth hormone or placental lactogen.

5. The method of claim 4 wherein said hormone receptor is growth hormone receptor, prolactin receptor or placental lactogen receptor.

6. The method of claim 1 wherein said mammal is selected from the group consisting of primate, ungulate, bovine, porcine, ovine, equine, feline, canine and rodentia.

7. A method of claim 5 wherein said mammal is human, said growth hormone is human growth hormone and said prolactin receptor is human prolactin receptor.

8. The method of claim 7 wherein said human growth hormone is native human growth hormone and the amino acid replaced is $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$.

9. The method of claim 8 wherein said other amino acid replacing $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$ is alanine.

10. The method of claim 4 wherein said placental lactogen hormone is human placental lactogen and said amino acid replaced is $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$.

11. The method of claim 10 wherein said other amino acid replacing $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$ is alanine.

12. A human growth hormone variant wherein $histidine_{21}$ of native human growth hormone is replaced by an amino acid other than glutamate, aspartate or cysteine.

13. The human growth hormone variant of claim 12 wherein said $histidine_{21}$ is replaced by alanine.

14. The human growth hormone variant of claim 13 further comprising replacing $histidine_{18}$ and $glutamate_{174}$ of native human growth hormone with an amino acid other than histidine, glutamate, aspartate or cysteine.

15. The human growth hormone variant of claim 14 wherein said $histidine_{18}$ and $glutamate_{174}$ are replaced by alanine.

16. A human placental lactogen variant wherein $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$ of native human placental lactogen is replaced by an amino acid other than histidine, glutamate, aspartate or cysteine.

17. The human placental lactogen variant of claim 16 wherein $histidine_{18}$, $histidine_{21}$ and $glutamate_{174}$ are all replaced by alanine.

18. A mammalian growth hormone variant excluding human growth hormone wherein the amino acid corresponding to human growth hormone amino acid $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$ is replaced by an amino acid other than histidine, glutamate, aspartate or cysteine.

19. The variant of claim 18 wherein said amino acid replacing the amino acid corresponding to $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$ is alanine.

20. The variant of claim 19 wherein said growth hormone is selected from the group consisting of: bovine, porcine, ovine, equine, feline, canine or rodentia growth hormone.

21. A method of stimulating a lactogenic response in a non-human mammal comprising:

a) administering to the mammal a therapeutically effective amount of a mammalian growth hormone wherein said mammalian hormone amino acid sequence contains amino acids corresponding to human growth hormone amino acids histidine$_{18}$, histidine$_{21}$ and glutamate$_{174}$; and

b) maintaining a physiological zinc ion concentration required for said mammalian growth hormone to bind to prolactin receptor wherein a lactogenic response is elicited.

22. The method of claim 21 wherein said total physiological zinc ion concentration is maintained between about 0.5 and 100.0 µmolar.

23. A method of stimulating a lactogenic response in a human comprising administering to the human a therapeutically effective amount of human growth hormone while maintaining a physiological zinc ion concentration required for said human growth hormone to bind to prolactin receptor wherein a lactogenic response is elicited.

24. The method of claim 23 wherein said total physiological zinc ion concentration is maintained between about 0.50 and 100.0 µmolar.

25. A method of stimulating a somatogenic response in a human comprising administering to the human a therapeutically effective amount of a human growth hormone variant in which the zinc binding site required for human growth hormone binding to prolactin receptor has been deleted.

26. The method of claim 25 wherein said variant has histidine$_{18}$, histidine $_{21}$ or glutamate $_{174}$ of native human growth hormone replaced by an amino acid other than glutamate, aspartate, histidine or cysteine.

27. The method of claim 26 wherein histidine$_{18}$, histidine$_{21}$ or glutamate$_{174}$ has been replaced by alanine.

28. A method of screening for variants of a mammalian polypeptide hormone thought to contain a metal ion binding site wherein the presence of a metal ion in said metal ion binding site determines said hormone's affinity for a hormone receptor in a mammal comprising:

a) incubating a solution containing a chelating agent and a mammalian polypeptide hormone variant suspected of containing a metal ion binding site;

b) contacting said incubated mammalian polypeptide hormone with a hormone receptor; and

c) detecting the formation of a polypeptide hormone-receptor complex.

29. The method of claim 28 wherein said metal ion is selected from the group consisting of zinc, iron, nickel, copper, magnesium, manganese, cobalt, calcium or selenium.

30. The method of claim 28 wherein said variant mammalian polypeptide hormone is a variant of growth hormone or placental lactogen.

31. The method of claim 28 wherein said mammal is selected from the group consisting of human, bovine, porcine, ovine, equine, feline, canine and rodentia.

32. A method of claim 30 wherein said mammal is human, said variant hormone is a human growth hormone variant and said receptor is human prolactin receptor.

33. A method of claim 32 wherein said variant is a human growth hormone variant wherein the amino acid replaced is $Histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$.

34. A method of claim 33 wherein said amino acid replacing $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$ is alanine.

35. A method of claim 30 wherein said mammal is human, said variant hormone is a human placental lactogen variant and said receptor is human prolactin receptor.

36. A method of claim 35 wherein said variant is a human placental lactogen variant wherein the amino acid replaced is $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$ corresponding to the amino acids in native human growth hormone.

37. A method of claim 36 wherein said amino acid replacing $histidine_{18}$, $histidine_{21}$ or $glutamate_{174}$ is alanine.

38. A mammalian prolactin binding protein variant comprising soluble prolactin binding protein.

39. The variant of claim 38 wherein said soluble prolactin binding protein is human prolactin binding protein.

40. The variant of claim 39 wherein $histidine_{188}$ is replaced by an amino acid other than histidine, glutamate, aspartate or cysteine.

41. The variant of claim 40 wherein $histidine_{188}$ is replaced by alanine.

42. A method of using the variant of claim 38 comprising administering to a mammal a therapeutic amount of said variant.

43. The method of claim 42 further comprising administering said variant in the presence of a therapeutic amount of growth hormone.

44. The method of claim 43 further comprising administering in the presence of a total physiological zinc ion concentration of between 0.5 and 100.0 μmolar.

45. The method of claim 43 wherein said variant is human soluble prolactin binding protein and said growth hormone is human growth hormone.

46. A DNA sequence encoding the human growth hormone variant of claim 12.

47. The DNA sequence of claim 46 wherein said variant contains alanine in place of $histidine_{18}$, $histidine_{21}$ and $glutamate_{174}$.

48. A DNA sequence encoding soluble human prolactin receptor.

49. The DNA sequence of claim 48 wherein said human prolactin receptor encoded contains an amino acid substitution at $histidine_{188}$.

50. The DNA sequence of claim 49 wherein said amino acid substitution at $histidine_{188}$ is alanine.

51. A DNA sequence encoding human growth hormone binding protein wherein $asparagine_{218}$ is replaced by an amino acid selected from the following: histidine, glutamic acid, asparatic acid and cysteine.

52. The DNA sequence of claim 51 wherein said amino acid selected is histidine.

53. Mammalian growth hormone binding protein wherein another amino acid is inserted in place of an amino acid corresponding to $asparagine_{218}$ in human growth hormone binding protein.

54. The mammalian growth hormone binding protein of claim 53 wherein said amino acid inserted is selected from alanine, histidine, glutamate, asparate and cysteine.

55. The mammalian growth hormone binding protein of claim 54 wherein said amino acid selected is histidine.

56. The mammalian growth hormone binding protein of claim 55 wherein said protein is human growth hormone binding protein.

57. A pharmaceutical formulation comprising mammalian growth hormone, mammalian growth hormone binding protein and zinc, wherein said mammalian growth hormone binding protein contains an amino acid substitution with histidine, glutamate, aspartate or cysteine.

58. The pharmaceutical formulation of claim 57 wherein said mammalian growth hormone is human growth hormone, said mammalian growth hormone binding protein is human growth hormone binding protein wherein asparagine$_{218}$ has been replaced by histidine.

59. A method of modifying a mammalian polypeptide hormone-receptor complex not containing a metal ion binding site to contain a metal ion binding site comprising:

a) determining the amino acid sequence of a polypeptide hormone or polypeptide hormone receptor not containing a metal ion binding site wherein said determined amino acid sequence has regions of homology with a polypeptide hormone or receptor having a metal ion binding site; and

b) modifying the amino acid sequence of said mammalian polypeptide hormone or hormone receptor not containing a metal ion binding site to contain one or more amino acids analogous to said polypeptide hormone or hormone receptor containing a metal ion binding site.

60. The method of claim 59 wherein said metal ion is zinc.

61. The method of claim 60 wherein said polypeptide hormone is growth hormone and said polypeptide hormone receptor is growth hormone binding protein.

62. An expression host transformed with a DNA sequence selected from the group consisting of:

a) a DNA sequence encoding a growth hormone variant wherein $histidine_{21}$ of human growth hormone is replaced by an amino acid other than glutamate, aspartate or cysteine; a DNA sequence encoding a soluble human prolactin receptor;

b) a DNA sequence encoding a soluble human prolactin receptor which contains an amino acid substitution at $histidine_{188}$ other than glutamate, aspartate or cysteine;

c) a DNA sequence encoding a soluble human prolactin receptor wherein $histidine_{188}$ is replaced by alanine; and

d) a DNA sequence encoding a human growth hormone binding protein wherein $asparagine_{218}$ is replaced by histidine.

63. A human growth hormone variant comprising $arginine_{64}$ and $aspartate_{171}$ substituted by alanine.

64. A human growth hormone variant comprising $lysine_{168}$ and $glutmate_{174}$ substituted by alanine.

65. A human growth hormone variant comprising $lysine_{172}$ and $glutmate_{174}$ substituted by alanine.

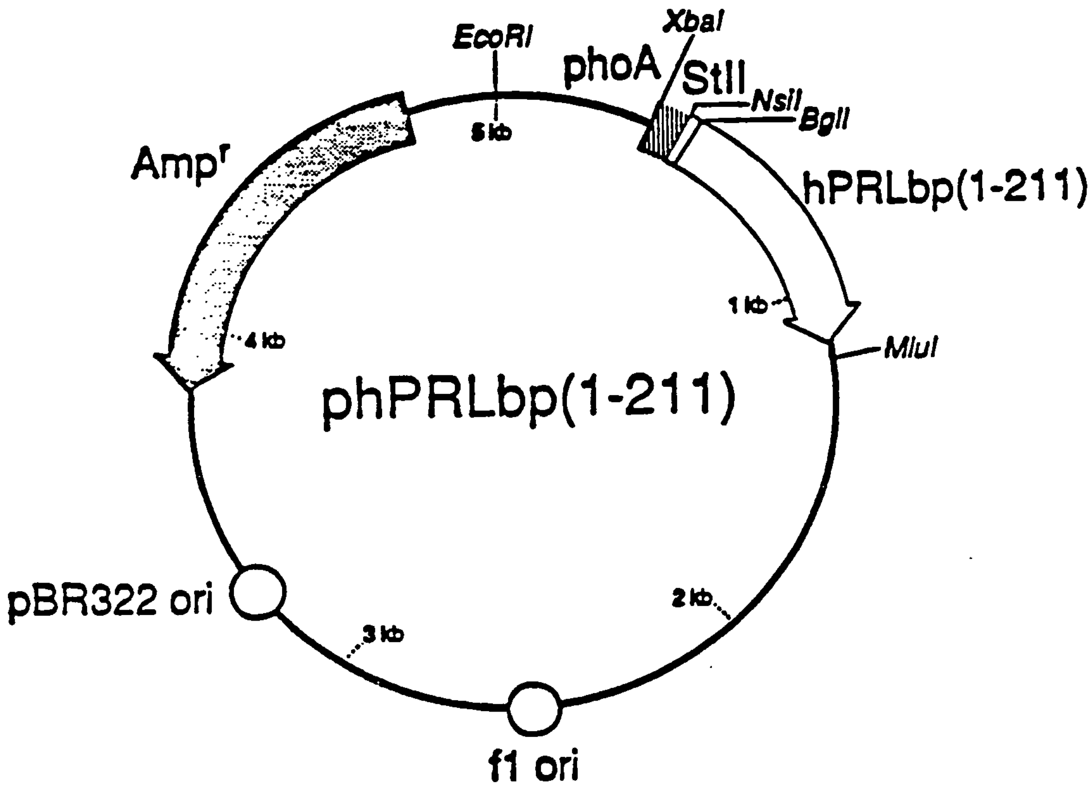
EcoRI
XbaI
phoA
StII
NsiI
BglI
Amp^r
5 kb
hPRLbp(1-211)
1 kb
MluI
4 kb
phPRLbp(1-211)
pBR322 ori
2 kb
3 kb
f1 ori

FIG 1A

FIG 1B

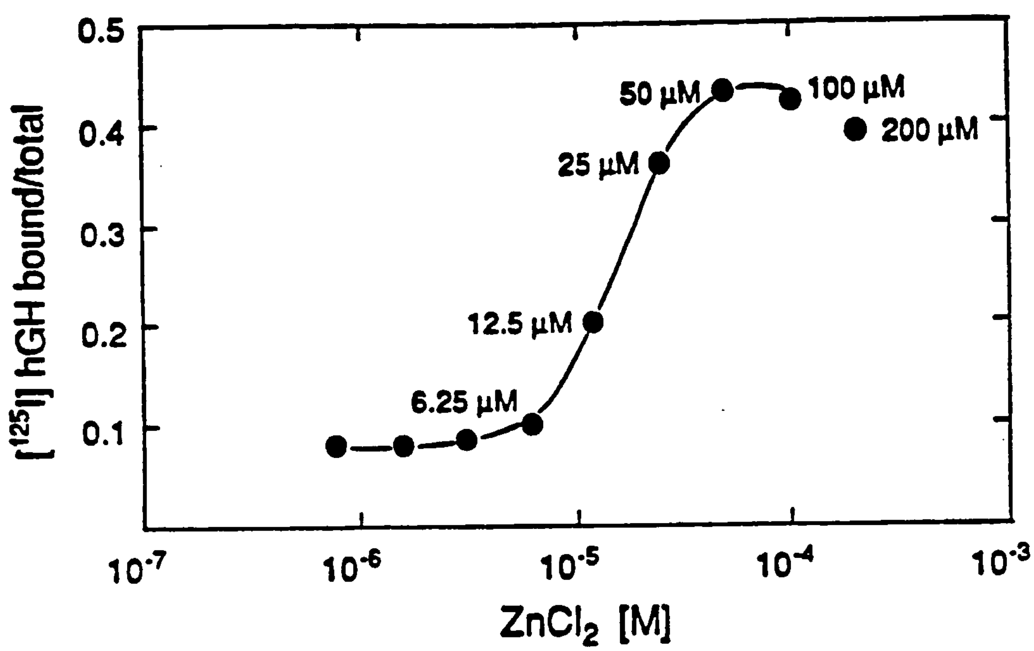
0.5
0.4
0.3
0.2
0.1
[125I] hGH bound/total
50 µM
100 µM
200 µM
25 µM
12.5 µM
6.25 µM
10-7
10-6
10-5
10-4
10-3
ZnCl2 [M]

FIG 2

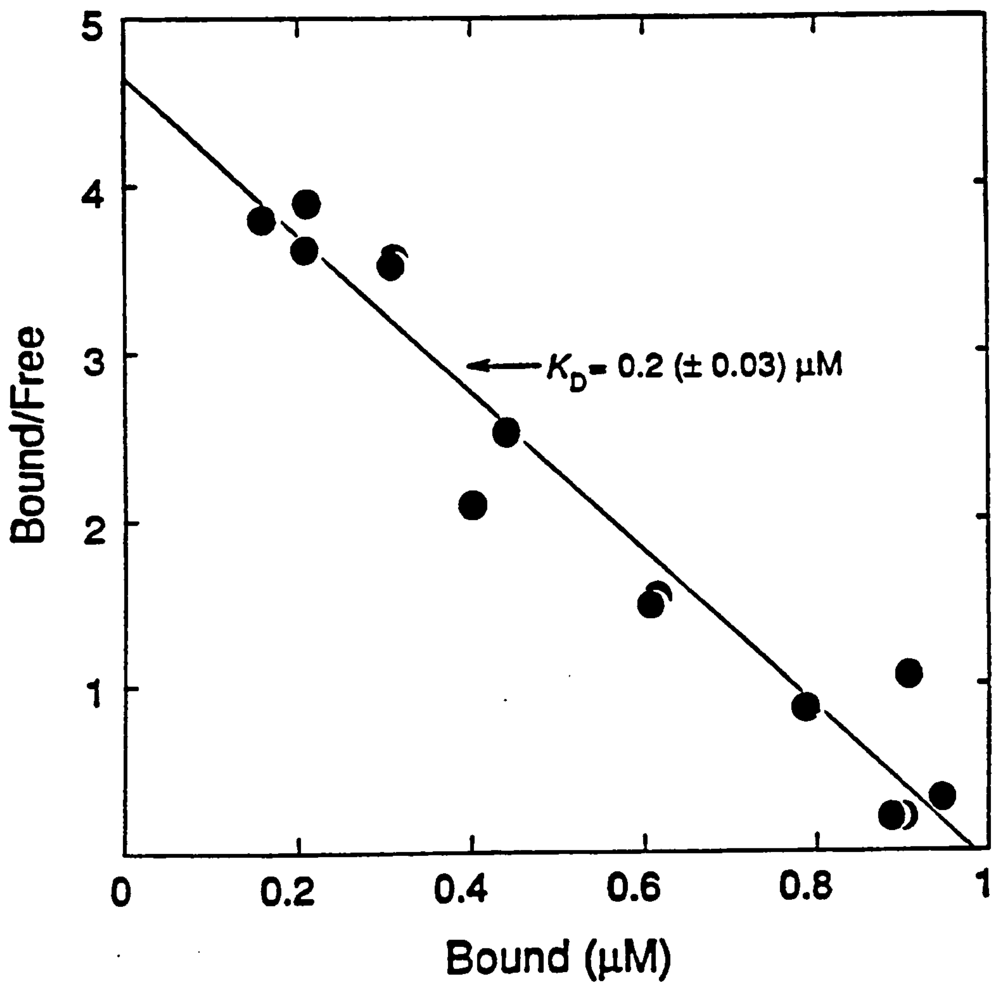

FIG 3

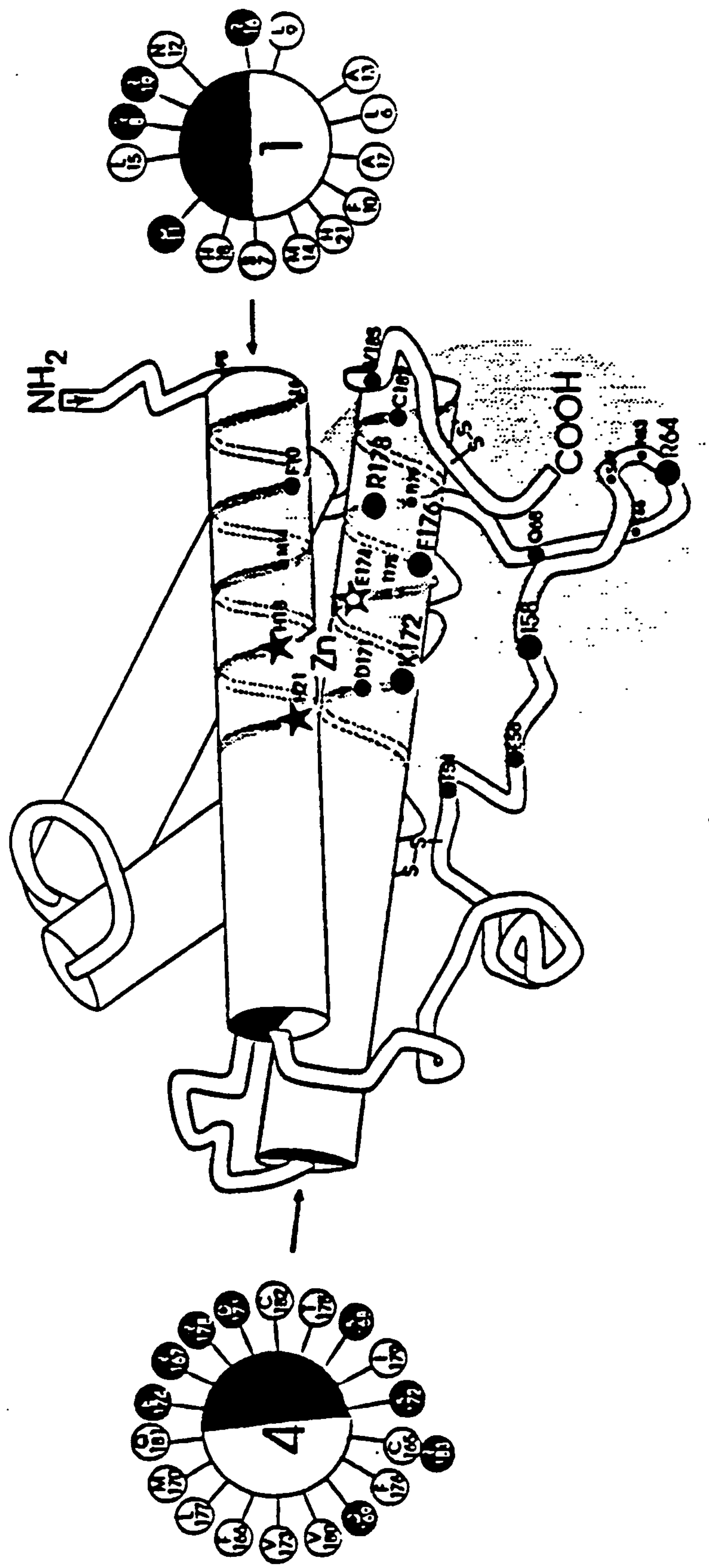

FIG 4

```
                10        20        30        40        50
shghr   FSGSEATAAILSRAPWSLQSVNPGLKTNSSKEPKFTKCRSPERETFSCHW
                                    ..  * . *****..***.* *
shprlr                            QLPPGKPEIFKCRSPNKETFTCWW
                                          10        20

                60        70        80        90       100
shghr   TDEVHHGTKNLGPIQLFYTRRNTQEWTQEWKECPDYVSAGENSCYFNSSF
            . *        * * .*. .   .   *****...* *** *   .
shprlr  RPGTDGGLP--TNYSLTY-HREGETLMH---ECPDYITGGPNSCHFGKQY
            30          40            50        60

               110           120       130       140
shghr   TSIWIPYCIKLTS----NGGTVDEKCFSVDEIVQPDPPIALNWTLLNVSL
        **.*  * . . .     ..  **    *  *******. *   . .
shprlr  TSMWRTYIMMVNATNQMGSSFSDELYVDVTYIVQPDPPLELAVEVKQP--
        70        80        90       100       110

         150       160       170       180       190
shghr   TGIHADIQVRWEAPRNADIQKGWMVLEYELQYKEVNETKWKMMDPILTTS
         .  . . ..*.*   *.. **  * **.. *  . . * .  .   *
shprlr  EDRKPYLWIKWSPPTLIDLKTGWFTLLYEIRLKPEKAAEWEIHFAGQQTE
         120       130       140       150       160

         200       210       220       230
shghr   VPVYSLKVDKEYEVRVRSKQRNSGNYGEFSEVLYVTLPQMSQ
          . **  .. * *.** *  . *  .  *   .. .*
shprlr  FKILSLHPGQKYLVQVRCK-PDHGYWSAWSPATFIQIPSDFTMNDT
         170       180        190       200       210
```

FIG 5

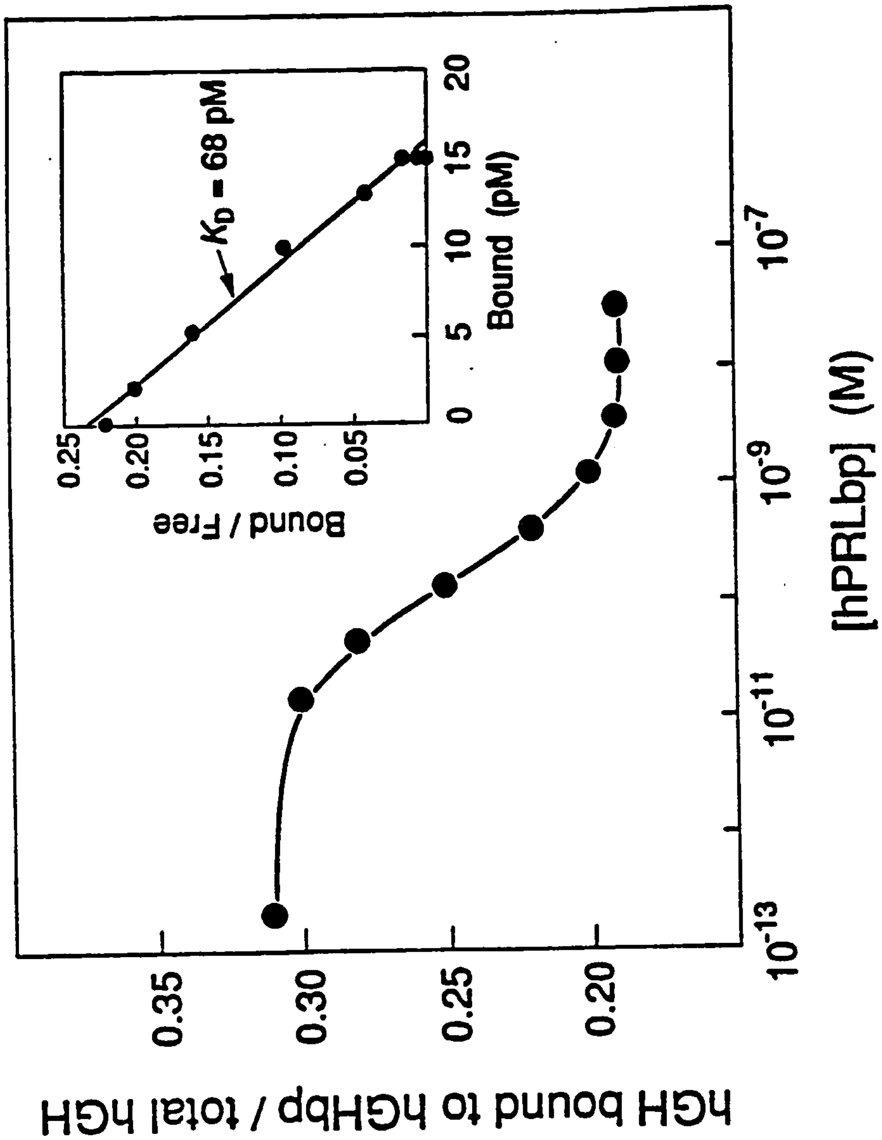
hGH bound to hGHbp / total hGH
0.35
0.30
0.25
0.20
10^{-13}
10^{-11}
10^{-9}
10^{-7}
[hPRLbp] (M)
Bound / Free
0.25
0.20
0.15
0.10
0.05
0
5
10
15
20
Bound (pM)
$K_D = 68$ pM

FIG 6

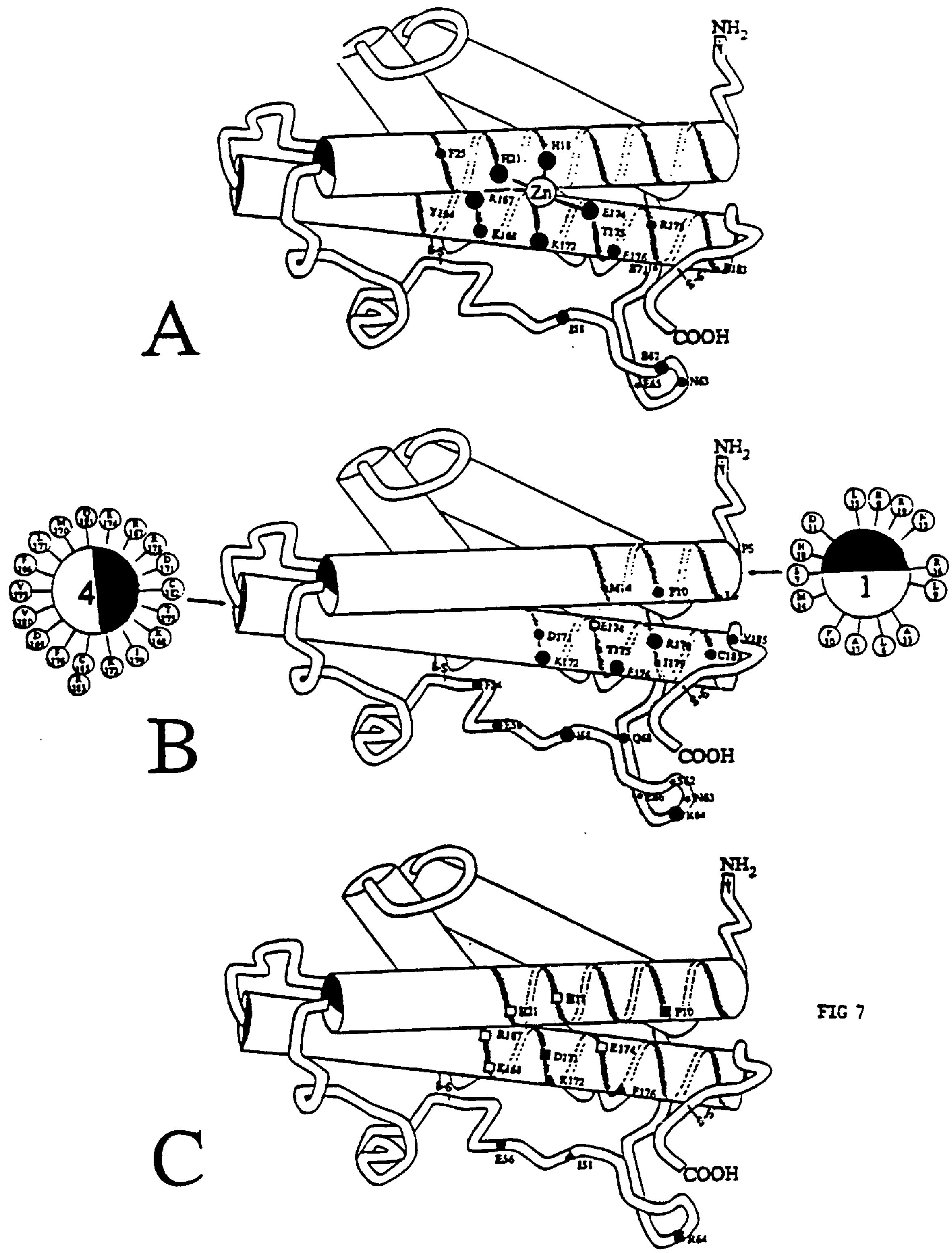

FIG 7

```
GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC 50

TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT 100

GAACTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT 150

TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG 200

GGGCGCTGTA CGAGGTAAAG CCCGATGCCA GCATTCCTGA CGACGATACG 250

GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA 300

AAAAGTTAAT CTTTTCAACA GCTGTCATAA AGTTGTCACG GCCGAGACTT 350

ATAGTCGCTT TGTTTTTATT TTTTAATGTA TTTGTAACTA GTACGCAAGT 400

TCACGTAAAA AGGGTATCTA GAGGTTGAGG TGATTTT    ATG AAA 443
                                            Met Lys
                                              1

AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT 482
Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
         5                  10                  15

TCT ATT GCT ACA AAT GCC TAT GCA TTC CCA ACT ATA CCA 521
Ser Ile Ala Thr Asn Ala Tyr Ala Phe Pro Thr Ile Pro
                 20                  25

CTA AGT CGA CTA TTC GAT AAC GCT ATG CTT CGG GCC CAT 560
Leu Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His
     30                  35                  40

CGT CTT CAT CAG CTA GCC TTT GAC ACC TAC CAG GAG TTT 599
Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu Phe
             45                  50

GAA GAG GCC TAT ATC CCC AAG GAA CAG AAG TAT TCA TTC 638
Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe
 55                  60                  65

CTG CAG AAC CCC CAG ACC TCC CTC TGT TTC TCA GAA TCG 677
Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser
         70                  75                  80
```

FIGURE 8 (1/7)

```
ATT CCG ACA CCC TCC AAT CGC GAG GAA ACA CAA CAG AAA 716
Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
                 85                  90

TCC AAC CTA GAG CTC CTC CGC ATA AGC TTG CTG CTC ATC 755
Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile
     95                 100                 105

CAG TCG TGG CTC GAG CCC GTG CAG TTC CTG AGG AGT GTC 794
Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val
            110                 115

TTC GCC AAC AGC CTG GTC TAC GGC GCC TCT GAT TCG AAC 833
Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn
120                 125                 130

GTG TAC GAC CTG CTG AAG GAC CTA GAG GAA GGG ATC CAA 872
Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln
        135                 140                 145

ACG CTG ATG GGG AGG CTG GAA GAT GGC AGC CCG CGG ACT 911
Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr
                150                 155

GGG CAG ATC TTC AAG CAG ACC TAC AGC AAG TTC GAC ACA 950
Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr
    160                 165                 170

AAC TCA CAC AAC GAT GAC GCA CTA CTC AAG AAC TAC GGG 989
Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly
            175                 180

CTG CTC TAC TGC TTC AGG AAG GAC ATG GAC AAG GTC GAG 1028
Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu
185                 190                 195

ACA TTC CTG CGC ATC GTG CAG TGC CGC TCT GTG GAG GGC 1067
Thr Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly
        200                 205                 210

AGC TGT GGC TTC T AGCTGCCCAG CTTTAATGCG GTAGTTTATC 1110
Ser Cys Gly Phe
            214

ACAGTTAAAT TGCTAACGCA GTCAGGCACC GTGTATGAAA TCTAACAATG 1160

CGCTCATCGT CATCCTCGGC ACCGTCACCC TGGATGCTGT AGGCATAGGC 1210

TTGGTTATGC CGGTACTGCC GGGCCTCTTG CGGGATATCG TCCATTCCGA 1260
```

FIGURE 8 (2/7)

CAGCATCGCC AGTCACTATG GCGTGCTGCT AGCGCCGCCC TATACCTTGT 1310

CTGCCTCCCC GCGTTGCGTC GCGGTGCATG GAGCCGGGCC ACCTCGACCT 1360

GAATGGAAGC CGGCGGCACC TCGCTAACGG ATTCACCACT CCAAGAATTG 1410

GAGCCAATCA ATTCTTGCGG AGAACTGTGA ATGCGCAAAC CAACCCTTGG 1460

CAGAACATAT CCATCGCGTC CGCCATCTCC AGCAGCCGCA CGCGGCGCAT 1510

CTCGGGCAGC GTTGGGTCCT GGCCACGGGT GCGCATGATC GTGCTCCTGT 1560

CGTTGAGGAC CCGGCTAGGC TGGCGGGGTT GCCTTACTGG TTAGCAGAAT 1610

GAATCACCGA TACGCGAGCG AACGTGAAGC GACTGCTGCT GCAAAACGTC 1660

TGCGACCTGA GCAACAACAT GAATGGTCTT CGGTTTCCGT GTTTCGTAAA 1710

GTCTGGAAAC GCGGAAGTCA GCGCCCTGCA CCATTATGTT CCGGATCTGC 1760

ATCGCAGGAT GCTGCTGGCT ACCCTGTGGA ACACCTACAT CTGTATTAAC 1810

GAAGCGCTGG CATTGACCCT GAGTGATTTT TCTCTGGTCC CGCCGCATCC 1860

ATACCGCCAG TTGTTTACCC TCACAACGTT CCAGTAACCG GGCATGTTCA 1910

TCATCAGTAA CCCGTATCGT GAGCATCCTC TCTCGTTTCA TCGGTATCAT 1960

TACCCCCATG AACAGAAATT CCCCCTTACA CGGAGGCATC AAGTGACCAA 2010

ACAGGAAAAA ACCGCCCTTA ACATGGCCCG CTTTATCAGA AGCCAGACAT 2060

TAACGCTTCT GGAGAAACTC AACGAGCTGG ACGCGGATGA ACAGGCAGAC 2110

FIGURE 8 (3/7)

ATCTGTGAAT CGCTTCACGA CCACGCTGAT GAGCTTTACC GCAGCATCCG 2160

GAAATTGTAA ACGTTAATAT TTTGTTAAAA TTCGCGTTAA ATTTTTGTTA 2210

AATCAGCTCA TTTTTTAACC AATAGGCCGA AATCGGCAAA ATCCCTTATA 2260

AATCAAAAGA ATAGACCGAG ATAGGGTTGA GTGTTGTTCC AGTTTGGAAC 2310

AAGAGTCCAC TATTAAAGAA CGTGGACTCC AACGTCAAAG GGCGAAAAAC 2360

CGTCTATCAG GGCTATGGCC CACTACGTGA ACCATCACCC TAATCAAGTT 2410

TTTTGGGGTC GAGGTGCCGT AAAGCACTAA ATCGGAACCC TAAAGGGAGC 2460

CCCCGATTTA GAGCTTGACG GGGAAAGCCG GCGAACGTGG CGAGAAAGGA 2510

AGGGAAGAAA GCGAAAGGAG CGGGCGCTAG GGCGCTGGCA AGTGTAGCGG 2560

TCACGCTGCG CGTAACCACC ACACCCGCCG CGCTTAATGC GCCGCTACAG 2610

GGCGCGTCCG CATCCTGCCT CGCGCGTTTC GGTGATGACG GTGAAAACCT 2660

CTGACACATG CAGCTCCCGG AGACGGTCAC AGCTTGTCTG TAAGCGGATG 2710

CCGGGAGCAG ACAAGCCCGT CAGGGCGCGT CAGCGGGTGT TGGCGGGTGT 2760

CGGGGCGCAG CCATGACCCA GTCACGTAGC GATAGCGGAG TGTATACTGG 2810

CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG 2860

GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCT 2910

CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT TCGGCTGCGG 2960

FIGURE 8 (4/7)

```
CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC 3010

AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA 3060

GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG GCTCCGCCCC 3110

CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT GGCGAAACCC 3160

GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC TCCCTCGTGC 3210

GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC CGCCTTTCTC 3260

CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA GGTATCTCAG 3310

TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG 3360

TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC 3410

CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG GTAACAGGAT 3460

TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG AAGTGGTGGC 3510

CTAACTACGG CTACACTAGA AGGACAGTAT TTGGTATCTG CGCTCTGCTG 3560

AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT CCGGCAAACA 3610

AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG CAGATTACGC 3660

GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT 3710

GACGCTCAGT GGAACGAAAA CTCACGTTAA GGGATTTTGG TCATGAGATT 3760

ATCAAAAAGG ATCTTCACCT AGATCCTTTT AAATTAAAAA TGAAGTTTTA 3810
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FIGURE 8 (5/7)

AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG TTACCAATGC 3860

TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC GTTCATCCAT 3910

AGTTGCCTGA CTCCCCGTCG TGTAGATAAC TACGATACGG GAGGGCTTAC 3960

CATCTGGCCC CAGTGCTGCA ATGATACCGC GAGACCCACG CTCACCGGCT 4010

CCAGATTTAT CAGCAATAAA CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG 4060

TGGTCCTGCA ACTTTATCCG CCTCCATCCA GTCTATTAAT TGTTGCCGGG 4110

AAGCTAGAGT AAGTAGTTCG CCAGTTAATA GTTTGCGCAA CGTTGTTGCC 4160

ATTGCTGCAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA TGGCTTCATT 4210

CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC CCCATGTTGT 4260

GCAAAAAAGC GGTTAGCTCC TTCGGTCCTC CGATCGTTGT CAGAAGTAAG 4310

TTGGCCGCAG TGTTATCACT CATGGTTATG GCAGCACTGC ATAATTCTCT 4360

TACTGTCATG CCATCCGTAA GATGCTTTTC TGTGACTGGT GAGTACTCAA 4410

CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG CTCTTGCCCG 4460

GCGTCAACAC GGGATAATAC CGCGCCACAT AGCAGAACTT TAAAAGTGCT 4510

CATCATTGGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG ATCTTACCGC 4560

TGTTGAGATC CAGTTCGATG TAACCCACTC GTGCACCCAA CTGATCTTCA 4610

GCATCTTTTA CTTTCACCAG CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA 4660

AAATGCCGCA AAAAAGGGAA TAAGGGCGAC ACGGAAATGT TGAATACTCA 4710

FIGURE 8 (6/7)

TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG TTATTGTCTC 4760

ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT 4810

TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGACGTCTAA GAAACCATTA 4860

TTATCATGAC ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT 4910

CTTCAA 4916

FIGURE 8 (7/7)